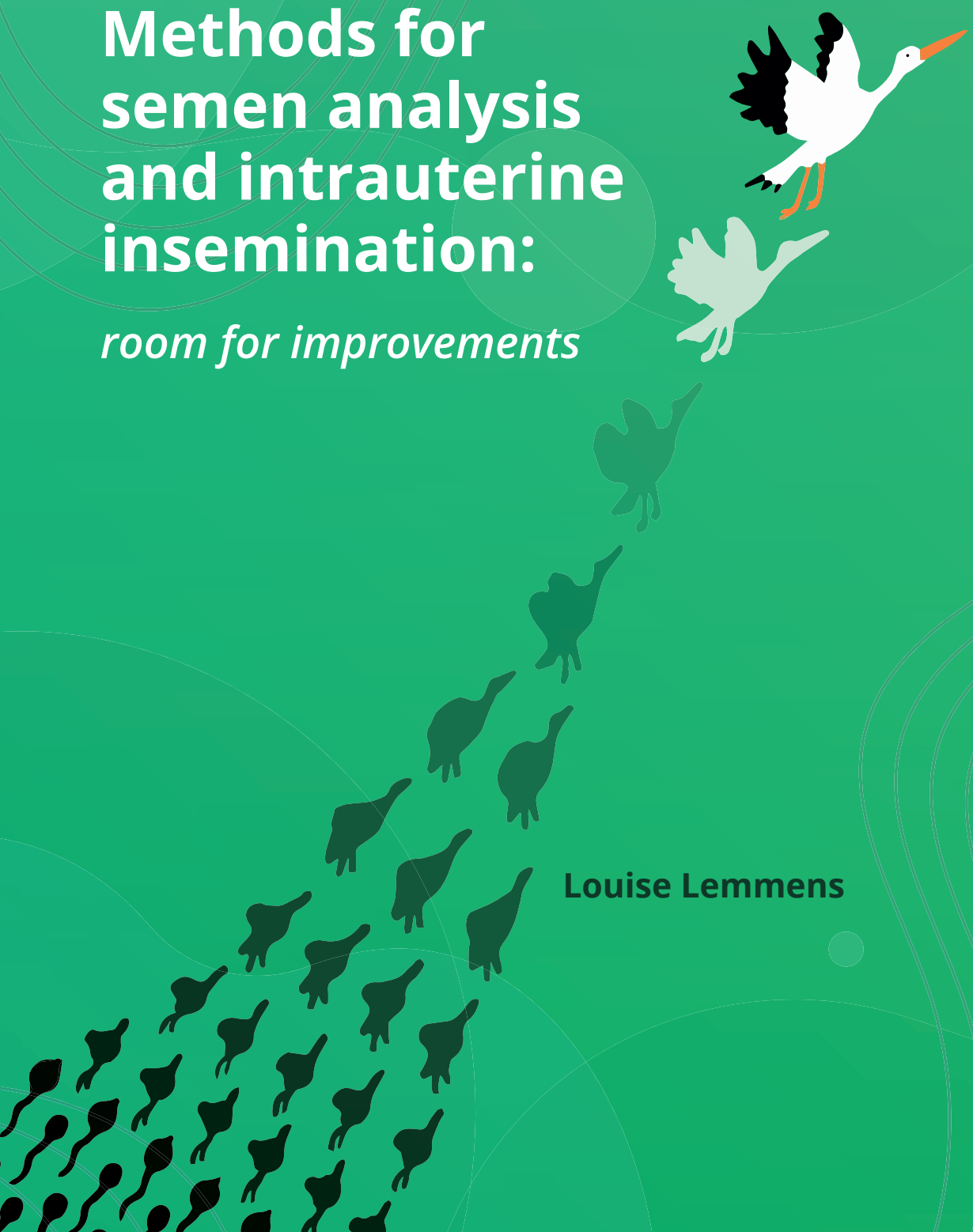
Methods for semen analysis and intrauterine insemination:

room for improvements



Louise Lemmens

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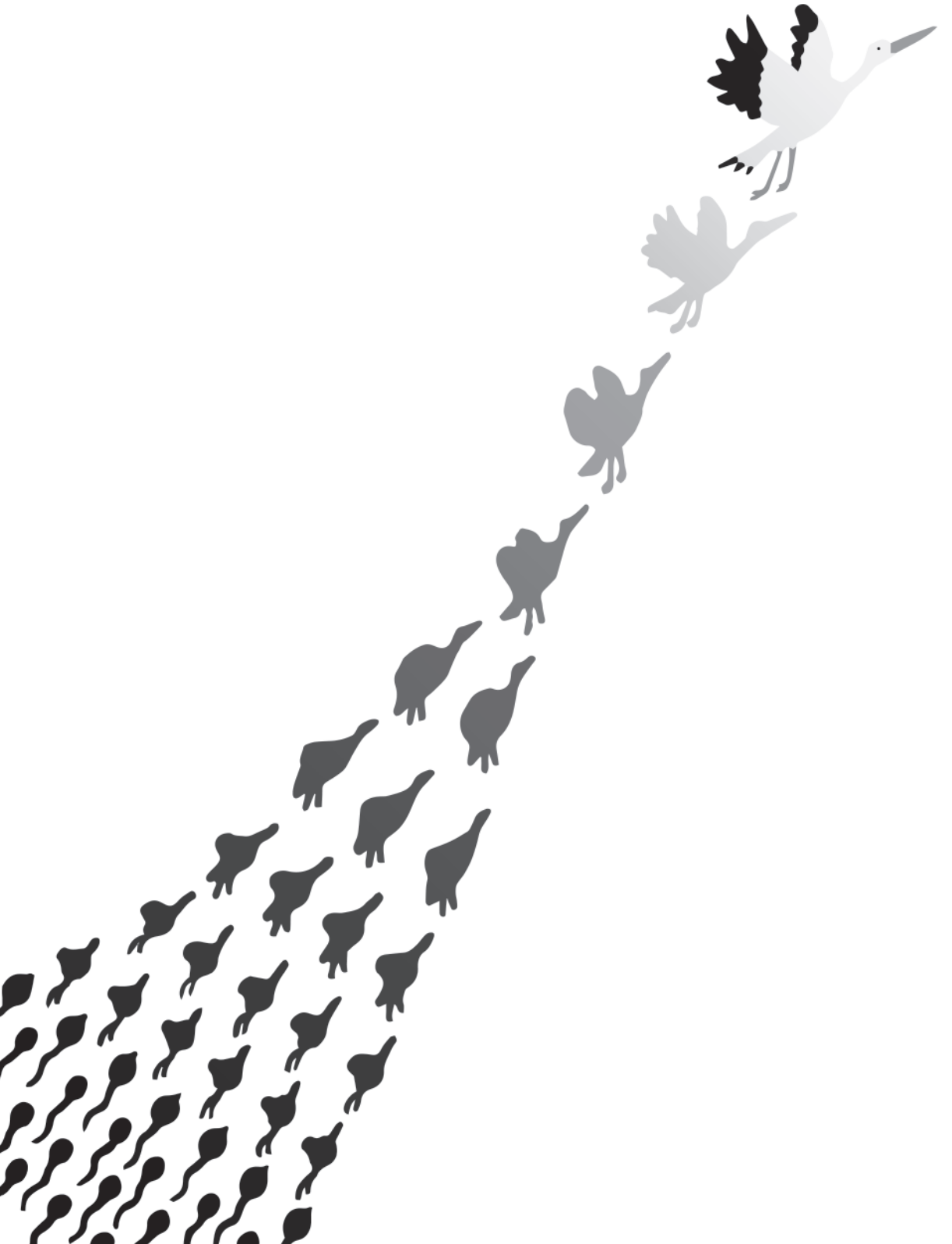

CHAPTER 1

General introduction and outline of this thesis

Infertility is defined as the inability of couples to achieve a clinical pregnancy within 12 months of regular unprotected intercourse (1). The inability of conceiving a child has a strong impact on couples, including societal repercussions and personal suffering (2). The prevalence of infertility varies across different regions, on average about 15% of the reproductive-aged couples are affected by infertility (1). Infertility is related to male factors, female factors (i.e. factors affecting or interfering with ovulation, fertilization or implantation), a combination of these or the cause of infertility remains unclear. Male factor infertility contributes to approximately 50% of all infertility couples and affects 8-12% of men in Europe (3) and can be related to spermatogenesis or fertilization problems.

Spermatogenesis and fertilization

Spermatogonia develop into spermatozoa during spermatogenesis, which is a complex process that takes place within the seminiferous epithelium in the testis. This structure consists of germ cells and radially-oriented supporting somatic cells called Sertoli cells. An overview of the anatomical units involved in spermatogenesis and the three major phases (i.e. spermacytogenesis, meiosis and spermiogenesis) is shown in Figure 1.

Spermatogonial stem cells are located near the basement membrane of the seminiferous epithelium and divide either in type A spermatogonia, to renew stem cell population, or in type B spermatogonia. Type B spermatogonia are concomitant to the spermatogenic differentiation pathway. Afterwards, these cells entry into meiosis, where each cell will be divided into four round haploid spermatids (4). During spermiogenesis, a round spermatid will develop into a highly specialized spermatozoon. This transformation includes five steps: formation of the acrosome, nuclear changes, development of the flagellum, reorganization of the cytoplasm and cell organelles, and the process of release from the Sertoli cell termed spermiation (5).

The sperm cells produced in the seminiferous epithelia are transported through the rete testis and stored in the tail of the epididymis. During storage in the epididymis, maturation of the spermatozoa takes place, which is needed for spermatozoa to be capable of motility. Many morphological and structural changes occur during maturation, one of them is the formation of disulfide bonds between protamines for further DNA condensation (6). Once ejaculation takes place, the motility of spermatozoa becomes activated and they are transported through the vas deferens, where they are mixed with secretion from the prostate. Thereafter, fluid from seminal vesicals and secretions from the bulbourethral glands are added (7). An overview of the male reproductive tract is shown in Figure 2. The total process of spermatogenesis including transport in the ductal system takes approximately 3 months.

Spermatogenesis is regulated by a complex integrated hormonal axis, known as the hypothalamic-pituitary-gonadal axis (see Figure 1). It all starts at the hypothalamus, where neurosecretory cells release gonadotropin-releasing hormone (GnRH). GnRH stimulates gonadotropin secretion by the pituitary gland, such as Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) (8). LH stimulates the testicular production of testosterone in the Leydig cells. Testosterone and FSH are crucial to the initiation and maintenance of spermatogenesis and they are necessary for maximal spermatogenic output (5). Circulating testosterone regulates the production and release of LH by the pituitary gland via a negative feedback mechanism. FSH stimulates the production of inhibins by the Sertoli cells, which results in negative feedback on the pituitary production of FSH secretion (9).

Spermatozoa enter during coitus the female genital tract in the anterior vagina, from where they swim quickly into the cervical canal. There they face the cervical mucus, which will become highly hydrated under the influence of estrogen to allow penetration of morphologically normal, motile sperm. After passage of the cervical mucus barrier, sperm are transported through the uterus. Transport is likely aided by contractile activity of the myometrium of the uterus. When sperm are subsequently transported through the uterotubal junction, they enter the tubal isthmus. The isthmus serves as a reservoir, where fertility of sperm is maintained until ovulation. At the time of ovulation, ovulatory factors like progesterone, induce sperm capacitation. Capacitation leads to sperm hyperactivation and prepares the sperm membrane to undergo the acrosome reaction by release of cholesterol from the sperm membrane. The hyperactivated sperm is released from the isthmus and will move through the Fallopian tubes, towards the oviductal ampulla (10).

The spermatozoa that reach the ovulated oocyte in the ampulla, surround it and try to force through the cumulus mass. When a spermatozoon reaches the zona pellucida, binding with a glycoprotein sperm receptor (zona pellucida protein 3) takes place. This allows the spermatozoon to undergo the acrosome reaction and to successful penetration of the zona pellucida, whereafter the cell membranes of the spermatozoon and oocyte fuse. Factors from the post-acrosomal region activate the oocyte, leading to the release of cortical granules. Membrane fusion immediately causes the zona to become impenetrable for other spermatozoa and the oocyte to resume meiosis, leading to the extrusion of a second polar body (7). Thus, of the millions of sperm inseminated at the female genital tract, only a few thousand arrive at the Fallopian tubes and only one will fertilize an oocyte. Two pronuclei are formed and brought together in the center of the oocyte by the action of sperm centrioles, which results in syngamy and the first mitotic division (11).

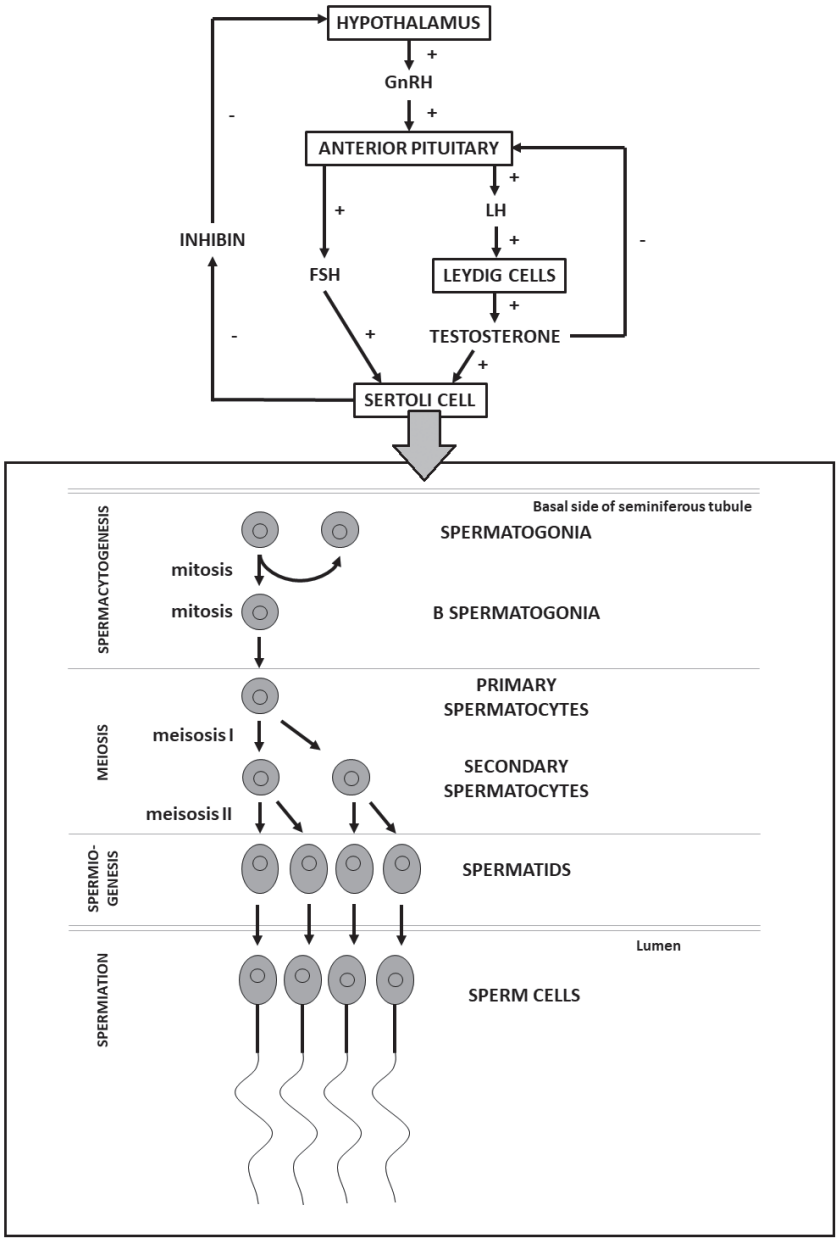

Figure 1. Overview of hypothalamic-pituitary-gonadal axis and the major phases of spermatogenesis

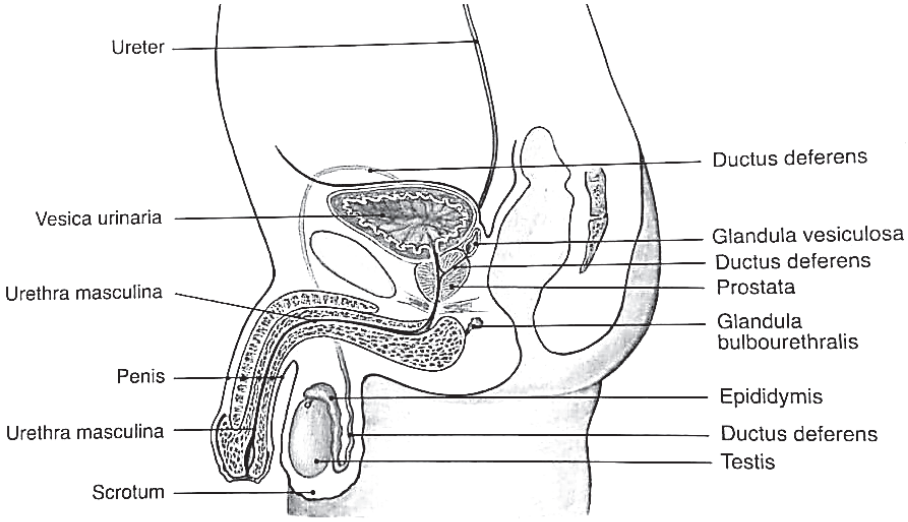

Figure 2. Anatomy of the male reproductive tract (32)

Causes of male infertility

Any defect in the complex process of spermatogenesis and fertilization can result in infertility. Disorders affecting the production of spermatozoa can mainly be divided in pretesticular, testicular and post-testicular causes of male factor infertility. In pretesticular dysfunction, the spermatogenesis is disturbed due to disorders of the hypothalamic-pituitary-gonadal axis. These endocrine disorders (i.e. disturbance of GnRH secretion, LH and FSH function or androgen function) have been identified in up to 20% of infertile men (12).

Testicular dysfunction is the most frequent cause of disturbed spermatogenesis and includes varicocele, genetics, cryptorchidism and exposure to gonadotoxins. Varicoceles are vascular abnormalities of the venous system of the testes. They are found in approximately 35% of men with primary infertility problems, but the exact mechanism of varicoceles causing infertility is still unknown. Moreover, in most cases it is unclear whether the varicoceles are actually the cause of the infertility. Cryptorchidism is abnormal descending of one or both testes before birth and is the most common congenital abnormality of male genitalia (13). Genetic disorders (such as deletions in the AZF region of the Y chromosome or Klinefelter syndrome) affect male infertility in various degrees, causing altered spermatogenesis, oligozoospermia, impaired normal development of the genital tract or decreased sperm motility and fertilization capacity. Examples of gonadotoxins and environmental factors affecting testicular functioning are

drugs, chemicals and excessive alcohol intake (12). Moreover, overexposure to other environmental factors may cause male infertility, such as heat and radiation.

Post-testicular deficiency includes ejaculatory dysfunction and obstruction of the excretory ductal system of sperm. Obstruction can occur along the epididymis, vas deferens or ejaculatory ducts. The most common cause of post-testicular infertility is epididymal obstruction, for example caused by previous infection or surgery. Moreover, vas deferens obstruction may be caused by an infection, vasectomy or hernia surgery (3). Ejaculatory dysfunction can be caused by neurologic, anatomic and psychologic conditions (for example diabetes mellitus and spinal cord injury) and can result in lack of emission ejaculation and retrograde ejaculation (12).

Standard semen analysis

Semen analysis is performed to evaluate male fertility. The complex processes of spermatogenesis and fertilization are described earlier, but female factors also influence the fertilization potential. This reflects the difficulties of developing a test that reports the direct correlation between semen quality and the chance that a spermatozoon fertilizes an oocyte. Abnormal values in semen analyses are, therefore, an indication for abnormal spermatogenesis or inadequate transport of semen and is not a direct measurement of male fertility.

The three main aspects assessed during standard semen analysis are sperm concentration, morphology and motility. Sperm concentration is defined as the number of spermatozoa per mL of semen. It is influenced by the volume of secretions produced by the prostate, seminal vesicles and bulbourethral glands, where spermatozoa are mixed with during ejaculation. Sperm concentration is, therefore, not a specific measure of testicular function, but it does relate to fertilization and pregnancy rates (14). The lower reference limit for sperm concentration is 15 million spermatozoa per mL, measurements below this level are defined as oligozoospermia (14). The lower reference limit for the total sperm number lies at 39 million spermatozoa per ejaculate, which in fact is a better measure of testicular function (15).

The morphology of a spermatozoon is considered as normal, when head, midpiece and tail have a normal shape according to defined criteria (Table 1). The head plays an important role in fertilization, the midpiece generates energy for swimming and the tail plays an important role in the propulsion system of the spermatozoon (7). In this way, the percentage of morphologically normal spermatozoa is a predictor for fertility. The strict criteria for sperm morphology was introduced in the late 1980s (16). A percentage of morphologically normal spermatozoa below 4% is considered as low (defined as teratozoospermia) (14).

Table 1. Criteria for spermatozoa to be considered as normal

Head	Midpiece	Tail
• Smooth, regularly countered • Oval in shape • Well-defined acrosomal region 40-70% of the head area • No large vacuoles in acrosomal region • ≤2 small vacuoles in acrosomal region <20% of sperm head • No vacuoles in post-acrosomal region	• Slender, regular and approximately the same length as sperm head • Major axis aligned with major axis of sperm head • Excess residual cytoplasm (i.e. when it exceeds 1/3 of sperm headsize)	• Uniform caliber along its length • Thinner than midpiece • Approximately 45 µm long (about 10 times head length) • No sharp angle indicative of a flagellar break

The motility of spermatozoa is graded based on their level of movement: spermatozoa moving actively are progressively motile, motile spermatozoa with absence of progression are non-progressively motile and spermatozoa with no movement are immotile. Earlier, progressively motile spermatozoa were also categorized as being rapid or slow (WHO 1999). Motility of spermatozoa is needed to move through the female genital tract in order to fertilize the oocyte. The lower reference limits are 32% of sperm with progressive motility and 40% for total motility (both progressively and non-progressively motile spermatozoa), measurements below these levels are defined as asthenozoospermia (14).

In addition to these three main aspects, assessment of pH (>7.5), volume (> 1.5 ml), viscosity and other factors (for example round cells, bacteria, color) is performed during semen analysis.

Classification

Semen analysis is an important requisite to evaluate male fertility. During anamnesis other factors should be taken into account as well, such as the duration of infertility, age of the couple and sexual, family, childhood, developmental, surgical and fertility history, and the possible exposure to gonadotoxins (17). Additionally, physical examination of the male and identification of possible genetic and endocrinologic factors may be determined.

The World Health Organization (WHO) defines male factor infertility as the presence of at least one abnormal level of the three main aspects assessed during semen analysis (i.e. sperm concentration, morphology or motility) or inadequate sexual or ejaculatory function (18). The most significant abnormalities of semen analysis are oligozoospermia, teratozoospermia and asthenozoospermia, or a combination of these. About 15% of infertile men are diagnosed with azoospermia, which is the complete absence of sperm in the semen. Azoospermia is categorized as obstructive azoospermia, where the genital tract is apparently obstructed since

endocrine and exocrine systems show no abnormalities, and non-obstructive azoospermia, where spermatogenesis is abnormal while serological levels of FSH and LH are elevated (19). The most common abnormalities of semen analysis are related to non-obstructive infertility.

Classification can be based on the reference values of the WHO (see section standard semen analysis), but other reference values are described as well. It is, for example, suggested to classify sperm parameters based on their value into more clinically relevant categories (i.e. normal, borderline and pathological (20), or infertile, indeterminate fertile and fertile (21)). Furthermore, classification is dependent on the clinical interpretation of the results (e.g. focusing on the healthiness of the spermatogenic process or on the probability of becoming pregnant). Taking female factors into account, classification of male factor infertility is relevant for selecting the treatment option for the infertile couple. The three main treatment options for infertility are intrauterine insemination (IUI), in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). During IVF, oocytes are collected and in-vitro fertilized by sperm. During ICSI, a single spermatozoon is injected directly into an oocytes cytoplasm. After fertilization, the embryo(s) are transferred into the uterus.

IUI was initially used as treatment option for couples with mild to moderate male factor infertility, but is nowadays also offered to couples with unexplained infertility (22). Originally, IVF was introduced as treatment option for couples suffering from tuba pathology. Over the years, however, it was offered to couples with all kind of indications, including severe male factor infertility (23). ICSI was introduced to treat couples with severe male factor infertility or even with azoospermia (i.e. using testicular sperm extraction) (24). Selecting the correct treatment option for infertile couples is of importance for optimizing pregnancy rates and, consequently, minimizing the number of required treatment cycles. The selection of either IUI, IVF or ICSI, depends on tubal patency and the pre-wash total progressively motile sperm count (TPMSC). For example, when TPMSC is below a certain level the choice of treatment shifts from IUI towards IVF or ICSI. The three main aspects of semen analysis (i.e. sperm concentration, morphology and motility) are assessed not only to evaluate testicular function, but also for selecting the best treatment option. It is, therefore, of importance that assessment of these factors is as adequate as possible.

Intrauterine insemination

Of all artificial reproductive technologies, IUI is one of the first treatment options for couples suffering from infertility and is the most simple and less invasive method. During IUI, washed and concentrated sperm are inserted through the cervical canal,

directly into the uterus, around the time of ovulation. The goal of IUI is to increase the number of sperm reaching the fallopian tube and, subsequently, increasing the chance of a sperm cell to fertilize an oocyte. To improve the chance of getting pregnant, IUI can also be carried out in combination with ovarian stimulation. Ovarian stimulation increases the number of oocytes available for fertilization (25). Although this increases the chance of getting pregnant, it also increases the chance of a multiple pregnancy (26). Multiple pregnancies cause more maternal and perinatal mortalities and morbidities compared to singleton pregnancies.

IUI is a less invasive and less expensive treatment option compared to IVF and ICSI. In couples with mild to moderate male factor infertility (e.g. ≥1 million motile spermatozoa), IUI is recommended as first-line therapy in many clinics (27). The rationale for this is that during the washing process of the semen sample, sperm with the best morphology and motility are concentrated and prepared for injection in the uterus. If IUI is offered to couples with mild to moderate infertility, clinical pregnancy rates range between 10-20% per cycle. Moreover, IUI is a cost-effective therapy in selected couples with mild male infertility, but also couples with cervical factor and immunological infertility (28). IUI is also an effective therapy in couples with unexplained infertility. Even though the exact difference in pregnancy rates is unclear, clinical studies showed that a combination of mild ovarian stimulation with IUI results in a higher chance of pregnancy in couples with unexplained infertility (29).

Shortcomings of semen analysis and IUI

Semen analysis has been performed for many years, as part of the diagnostic trajectory and for selecting the treatment option offered to an individual couple. However, semen analysis is characterized by a lack of standardized methodologies. This lack of standardization applies also to the different sperm preparation techniques used for assisted reproductive technologies (30, 31). The willingness to follow guideline recommendations was limited and a majority of fertility centers still use their own materials and methods (32). Moreover, provided recommendations were incomplete, due to a lack of supporting evidence (32). In order to realize standardization, more knowledge concerning semen analysis and IUI methods and their impact on pregnancy rates is necessary.

Another pitfall of semen analysis is the risk of subjectivity of the measurement, which can lead to variability of the results (i.e. intra- and inter-observer and inter-laboratory variability) (33). Strategies to overcome these problems related to semen analysis include training of the technicians performing semen analysis and the introduction of external quality control programs. Training including all different aspects of semen analysis resulted in a reduction of the variability (34),

but also in awareness of the need of standardization among the participating technicians (35,36). Quality control is a requisite to measure precision and accuracy of semen analysis results, which indirectly results in a lower level of variability of the three main aspects of semen analysis (i.e. sperm concentration, morphology and motility) (37) and improves standardization of the used methods (38).

Next to these technical shortcomings, another ongoing debate related to semen analysis is the value of sperm parameters as predictors of IUI outcome (31,39). The influence of sperm morphology assessment shows conflicting results on predicting pregnancy outcomes, especially since the introduction of ICSI. Since then, poor semen quality is usually a reason to select ICSI as treatment option, irrespective of sperm morphology (except for rare cases, such as globozoospermia). There is also disagreement on the predictive value of the total progressively motile sperm count (40) in IUI. These studies, however, were characterized by a lack of standardization.

The selection procedure in order to select the offered fertility treatment (i.e. IUI, IVF or ICS) has also been subject of discussion (41). Earlier research showed insufficient evidence to select one treatment option over the other, especially in couples with male factor infertility (42). Due to imprecise and inconsistent study designs, it was even not possible to report any differences in safety or effectiveness of fertility treatments (including timed intercourse, IUI, IVF and ICSI) (42). For these reasons, the British National Institute for Health and Care Excellence NICE) reduced the indications for IUI to sexual dysfunction, same sex relationship and selected conditions (43). Evidence for this recommendation, however, was reported as not sufficiently robust (44). However, others showed that IUI favors over IVF in many couples, based on clinical and economical evidence (45).

Despite these shortcomings, semen analysis and IUI are performed on a large scale, and conducted in many fertility clinics around the world (46,47). It is, therefore, important to improve the accuracy of semen analysis and the pregnancy outcomes of IUI. This will eventually result in improved counseling of infertile couples resulting in selecting the best treatment option for the couple.

Outline

Semen analysis and IUI are important requisites in the diagnosis and treatment of infertile couples. A lot of further research is needed for the optimization of semen analysis and IUI. This will eventually result in better counseling and treatment of infertile couples. The overall research question studied in this thesis is therefore: what tools and recommendations should be provided to infertility clinics as best practice for semen analysis and IUI in couples with male factor infertility? Answers

to this research question are described in chapters 2 -6. Their main goals are described below.

The importance of standardization in semen analysis and IUI protocols has been emphasized before (27). Since a lack of standardization might result in distinct selection of treatment option offered to the infertile couple, it might result in inter-laboratory variation in pregnancy rates (38). Chapter 2 provides, a literature overview of the association between pregnancy results of IUI and procedures used during the technical stage between semen collection and insemination. The provided recommendations aim to define best practice to realize standardization and can be a starting point for further research. To evaluate the implementation of the procedures recommended in chapter 2, chapter 3 describes Dutch actual care by summarizing the used procedures of IUI and semen analysis in Dutch fertility laboratories, as well as their effect on pregnancy results.

Next to optimizing IUI procedures, correct counseling of infertile couples and selection of the best treatment option for the individual couple is important. These steps are, among others, based on standard semen analysis results. It is, therefore, important that the predictive value of semen parameters for IUI success is investigated (28). This was studied in Chapter 4, where the value of sperm morphology and progressively motile sperm count to predict IUI pregnancy results is presented. In order to improve the relevance of semen analysis results, intra- and inter-observer variability and inter-laboratory variability should be reduced (39). Strategies to reduce variability in semen analysis results are introduction of external quality control programs (40) and training of technicians performing semen analysis (41). The impact of external quality control and standardized training in the Netherlands on semen analysis results is described in Chapter 5. Furthermore, an overview of the impact of a short on-site training on the semen analysis results in the Dutch EQC program is shown in Chapter 6.

This thesis concludes in Chapter 7 with a review of the main findings and a general discussion on these findings. Furthermore, recommendations for clinical practice and future research are provided.

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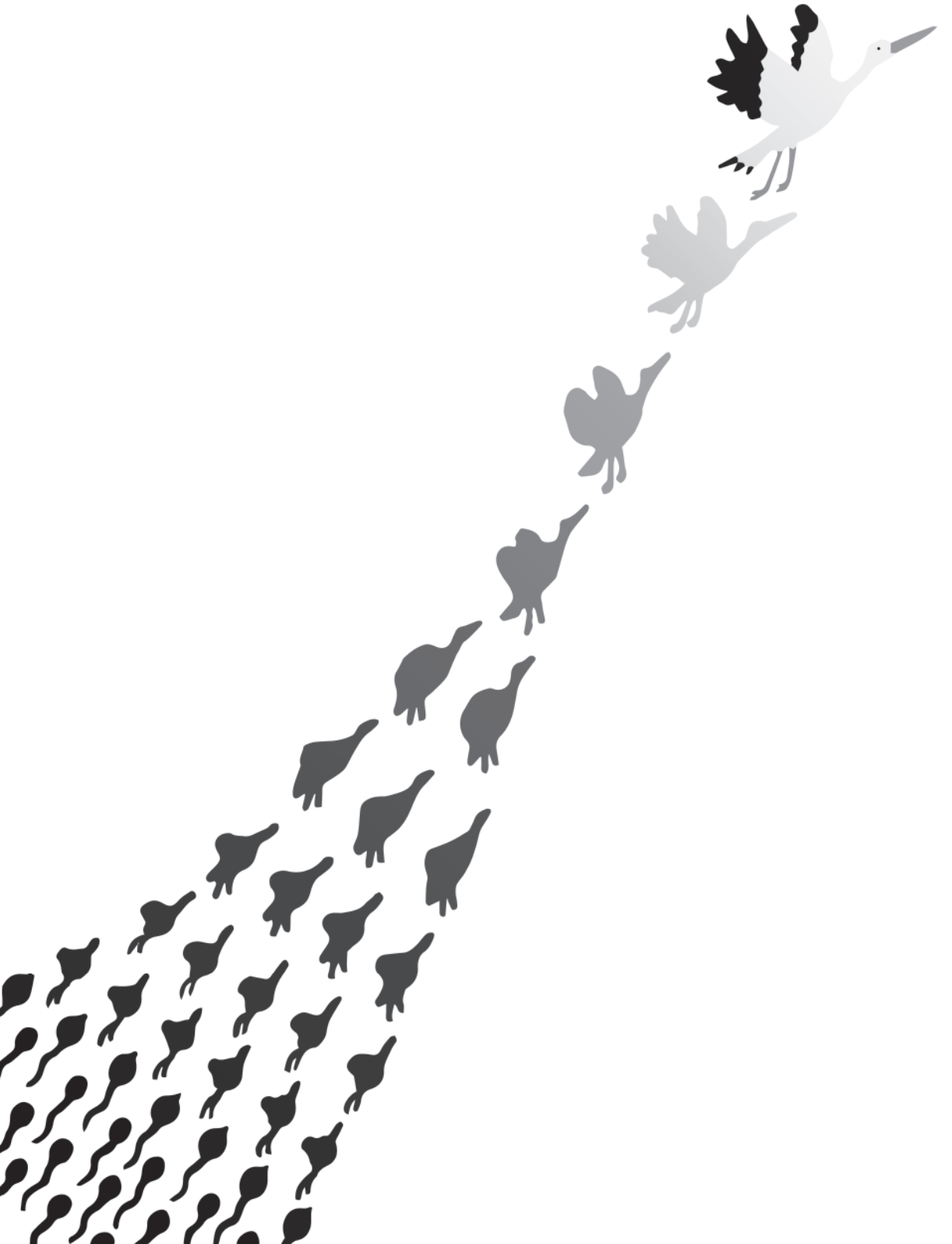

CHAPTER 2

Technical performance of intrauterine insemination: is it time for a change?

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ABSTRACT

Study question: Are the guidelines for the technical aspects of IUI (WHO, 2010) still in accordance with the current literature?

Summary answer: In general, the laboratory guidelines of the World Health Organization (WHO) are a suitable protocol, although the evidence is not always conclusive and some changes are advisable.

What is known already: Lack of standardization of the technical procedures required for IUI might result in inter-laboratory variation in pregnancy rates. Most centers still use their own materials and methods even though some guidelines are available.

Study design, size, duration: A structural review focusing on the association between pregnancy rates and the procedures of semen collection (e.g. ejaculatory abstinence, collection place), semen processing (e.g. preparation method, temperature during centrifugation/storage), insemination (e.g. timing of IUI, bed rest after IUI) and the equipment used.

Participants/materials, setting, methods: A literature search was performed in Medline and the Cochrane library. When no adequate studies of the impact of a parameter on pregnancy results were found, its association with sperm parameters was reviewed.

Main results and the role of chance: For most variables, the literature review revealed a low level of evidence, a limited number of studies and/or an inadequate outcome measure. Moreover, the comparison of procedures (i.e. semen preparation technique, time interval between semen, collection, processing and IUI) revealed no consensus about their results. It was not possible to develop an evidence-based, optimal IUI treatment protocol.

Limitations, reasons for caution: The included studies exhibited a lack of standardization in inclusion criteria and methods used.

Wider implications of the findings: This review emphasizes the need for more knowledge about and standardization of assisted reproduction technologies. Our literature search indicates that some of the recommendations in the laboratory guidelines could be adapted to improve standardization, comfort, quality control and to cut costs.

Introduction

At the moment, there is an ongoing discussion about the value of IUI. Recent Dutch studies showed a positive performance of the treatment especially in cases of mild andrological and unexplained infertility (1,2,3). On the other hand, the British guideline for infertility treatment strongly reduced the indications for IUI to sexual dysfunction, same sex relationship and special conditions (4). In this guideline, clinics are directed to apply in IVF or ICSI as a first line treatment in the majority of cases. However, the evidence cited to support this guideline was, not robust (5). Not only clinical, but also economical and financial evidence favors IUI over IVF in many cases (6). A lot of discussion is ongoing on this subject (7-9). Probably, for this reason, until now only a small proportion of clinics have made significant changes to their IUI practice (10) and IUI is still performed on a large scale worldwide and it is remains worthwhile to try to improve the outcome.

The IUI procedure can roughly be separated in three steps: diagnosis and indication, cycle preparation and the technical stage. The third step, including the whole process between semen collection and insemination, is barely included in guidelines. Only the World Health Organization (WHO) laboratory manual (11) attempts to describe the process. This description is incomplete, because parts of the pre- and post-laboratory stages are missing.

This structural review focuses on the technical phase of IUI and we check whether the present guidelines are in concordance with available literature. As the WHO manual (11) is the only international guideline that describes a protocol for semen collection, analysis and preparation, we used this guideline as the main reference point for our study.

Methods

The available literature on the following procedures or variables of IUI was reviewed: ejaculatory abstinence (EA), semen collection place, time intervals (i.e. between semen collection and semen processing, between semen processing and insemination, and between semen collection and insemination), semen preparation methods, centrifugation medium, centrifugation and storage temperature, timing of IUI, use of different disposables (e.g. catheters) and duration of bed rest after IUI.

A computerized search was carried out in Medline and in the Cochrane library. Key words for the search were 'intrauterine insemination', 'IUI' or 'artificial insemination'. Specific key words used for the individual variables included: 'ejaculatory abstinence', 'ejaculatory frequency', 'time interval', 'collection to processing', 'collection to IUI', 'processing to IUI', 'semen purification', 'semen preparation',

'semen separation', 'density gradient', 'swim up', 'wash', 'buffer', 'zwitterion', 'bicarbonate', 'HEPES', 'MOPS', 'TEST', 'medium', 'temperature', 'centrifugation', 'incubation', 'storage', 'timing', 'insemination timing', 'disposable', 'devices', 'tube', 'glove', 'pipette', 'catheter', 'collection container', 'bed rest', 'supine positioning', 'immobilization' and 'mobilization'. The titles and abstracts were screened to exclude citations considered as irrelevant, thereafter full texts of potentially eligible studies were reviewed. Articles published before 1 November 2016 in peer reviewed journals in the English language were included. The references and related citations of these articles were used to identify extra potential articles of interest. Studies reporting the impact of used laboratory procedures on sperm parameters or IUI pregnancy rates (PRs) were included.

The recommendations on technical aspects of IUI stated in the WHO guideline (11) were used as reference and compared with the results of the literature search. The results were arranged in an evidence-level structure as described by NICE (4). Finally, a summary is given of the recommendations, limitations of the available literature and knowledge gaps.

Ejaculatory abstinence

The WHO recommends an EA period of 2–7 days before semen collection (11), both for diagnostics and semen preparation. Although no explanatory literature is provided, studies on sperm parameters support this recommendation since an EA of 2–7 days resulted in a significantly higher semen volume (12-14) and total motile sperm count (TMSC) (15-17). On the contrary, a recent study reported a significant higher sperm motility for ejaculates of infertile men if they were produced within 40 min after an initial sample with <5 million motile spermatozoa (18). Moreover, an EA period of 0–2 days also resulted in higher percentages of morphologically normal spermatozoa (16,18,19).

The explanation of these observations can be found in the effect of reactive oxygen species (ROS). A certain level of ROS is required for the maturation of epididymal spermatozoa (20). Excessive ROS, however, can induce oxidative damage which negatively affects the fertilization potential of spermatozoa (14). The exposure time of spermatozoa to ROS is influenced in an EA time-dependent manner, thereby influencing the incidence of sperm DNA fragmentation, especially in infertile men (21,22). As a consequence, a shorter period of EA will result in higher PRs both in natural and IUI cycles, especially in sub-fertile men (23,24).

So far, the relationship between duration of EA and IUI PRs has been investigated in only two retrospective studies. These studies showed a negative impact of longer EA periods on PRs in cohorts of 372 (17) and 417 couples (15). These

studies reported highest PRs in the group with an EA up to 2 days and up to 3 days, respectively. In a retrospective pilot study, it was also found that in cases of oligozoospermia, the aggregation of consecutive ejaculates resulted in a higher PR (25). So, irrespective of a higher TMSC, the WHO recommendation of 2–7 days is debatable. A possible bias in the plea for a shorter EA is that these couples had intercourse shortly before insemination, thereby increasing the probability of a natural conception. More studies are needed to confirm these findings, both in normozoospermic and oligozoospermic men. For now, it can be advised to change the WHO recommendation into an EA period of maximum 3 days.

Time intervals

The time intervals between semen collection to processing, processing to insemination and semen collection to insemination have impact on IUI PRs (Table I). However, the WHO provided only a recommendation for the time interval between collection and processing (11). They stated that semen sample collection for IUI should preferably take place in a private room near the clinical laboratory, but when collection at home is preferred the semen should be delivered to the laboratory within 1 h after collection (while protected from extremes of temperature) (11).

When comparing PRs, a higher PR was reported when semen was collected in the clinic (26). Another study found no difference in PRs between collection at home and in the clinic (27). Furthermore, semen collection in the clinic led to a time interval that was on average 26 min shorter than collection at home (26). Nevertheless, no impact of time interval duration was found in a large study population (*n* = 633 cycles) (27). This was also shown in women treated with clomiphene citrate (*n* = 95 cycles) in another study (26). On the contrary, a shorter time interval (i.e. 15–30 min) resulted in a higher PR in a small group of women treated with human menopausal hormone (hMG; *n* = 37 cycles) (26). Lower PRs caused by longer time intervals, might be explained by decapacitating factors in the seminal plasma (28-30) or ROS-induced DNA damage (14,26).

Table I. Summary of findings reported by literature when comparing the impacts of time intervals on the IUI pregnancy rate

Time interval	Time intervals studied (min)	Study	Included couples (n)	Cause of infertility	Time interval with statistically significant highest pregnancy rates (min)
Semen production --> processing	15-30, 31-60, >60	Yavas and Selub (2004)	62 (132 cycles)	Female, male, unexplained and combination	15-30 *
	<30, 30-60, >60	Song *et al.* (2007)	335 (633 cycles)	Female cervical factor, male	No difference
Semen processing --> IUI	≤30,31-60, >60	Yavas and Selub (2004)	62 (132 cycles)	Female, male, unexplained and combination	≤30 and 31-60 *
	Not defined	Song *et al.* (2007)	335 (633 cycles)	Female cervical factor, male	No difference
	<30, 30-59, 60-89, 90-119, 120-180	Kilicdag *et al.* (2005)	- (1125 cycles)	Mild male, unexplained	≥30
	<40, 40-80, >80	Fauque *et al.* (2014)	709 (862 cycles)	Female, male, unexplained	40-80
	<60, about 24h	Jansen *et al.* (2016)	1135 (2154 cycles)	Female, male, unexplained	No difference
Semen production --> IUI	≤90, 91-120, >120	Yavas and Selub (2004)	62 (132 cycles)	Female, male, unexplained and combination	≤90 *
	Not defined	Song *et al.* (2007)	335 (633 cycles)	Female cervical factor, male	No difference

*results reported in couples with human menopausal gonadotropin (hMG)-treated women, no differences in couples with clomiphene citrate (CC)-treated women

- = not available in study

Regarding storage time after processing (i.e. time interval between processing and insemination), a shorter time interval was related to a lower proportion of premature sperm chromatin decondensation (31), to less sperm DNA fragmentation (32) and to a higher PR due to the storage time-dependent spontaneous acrosome reaction (32,33). In practice, however, no consensus was shown in reported ideal time intervals. PRs were comparable when IUI was performed within 30 min or after 31–60 min of storage, but decreased after >60 min, only in couples with hMG-treated women (26). Others reported highest clinical PRs in the groups with a storage time of 40–80 min (32) and >30 min (34). With another approach, another study (27), found no differences in in storage time intervals between a group of pregnant and non pregnant couples. Moreover, a recent study reported no difference in ongoing PRs between immediate insemination and insemination one day after semen processing (35).

Two retrospective studies evaluated the impact of the total time interval between semen collection and insemination. In one study, higher PRs were found when insemination took place within 90 min after semen collection (i.e. compared to 91–120 min and >120 min) (26), the other study found no differences (27).

In conclusion, literature on this subject is scarce and presented contradictory results. More information can be obtained in RCTs, but also in retrospective, well-designed multicenter studies, where standardized time intervals should be compared. For now, it is not possible to recommend one time interval over the other, nevertheless, the majority of the results propose to avoid long time intervals. Especially the time interval between semen collection and processing should not exceed 60 min, since no pregnancies were reported in this group (26,27).

Semen preparation methods

After semen production and liquefaction, it is necessary to separate sperm from the seminal plasma, thereby preventing uterine cramps, extended ROS formation and inhibition of fertilization (28,36). Many separation techniques have been described. Compared to the initial semen sample, the use of all these techniques resulted in significantly better semen parameters (37-39) and higher IUI PRs (40).

According to the WHO, the choice of semen preparation technique should be based on the nature of the semen sample (11). It is recommended to use swim-up in cases of normozoospermia, while density gradients should be the method of choice in other cases.

Density gradient centrifugation started with the use of PercollR. In 1996, however, PercollR was withdrawn from the clinical market, since it was stated that the polyvinylpyrrolidone (PVP)-coated silica in PercollR contained endotoxins (41). Since then, several endotoxin-free products with silane-coated silica particles were introduced. In first instance, research concentrated on comparing these new products to PercollR and conflicting results were found with respect to sperm motility and recovery rate (38,42-47). Despite these disagreements, silane-coated products are now widely used (48).

There is consensus that the swim-up technique resulted in lower recovery rates compared to density gradient centrifugation, making it suitable only in cases of normozoospermia (48,49). As swim-up selects spermatozoa based on their motility, one would expect that it would result in a high fraction of motile spermatozoa. Some studies, however, reported a comparable or even lower motility if swim-up was compared to gradients (50-52). The same is true for the percentage morphologically normal spermatozoa (50,53-55).

In practice, the clinical outcome of IUI is of more importance than the value of semen parameters. In 2007, a systematic Cochrane review (56) included six RCTs in their meta-analysis, comparing the effectiveness of density gradient techniques versus swim-up techniques and versus wash-only. They concluded that there is no evidence to choose for one technique over the other. The included studies, however, were characterized by low numbers of patients, diversity in the cause of infertility and diversity in the techniques that were compared. Only one study (57) included a larger study population (*n* = 363). Still, this study is of limited value, since five different techniques were studied in a population with all causes of infertility. Since Boomsma's review, only one suitable RCT has been performed. A significantly higher PR (both per cycle and per couple) was found using density gradient centrifugation (SpermGradR) compared to the swim-up technique, in couples with unexplained infertility (58). An overview of all studies is given in Table II (47,52,58-64). Additional studies with standardized patient inclusion criteria and study designs are necessary to confirm the results obtained from these studies.

pH buffer of washing and storage medium

To maintain an optimal pH level, the WHO recommends to select a buffer medium based on the used incubator: a zwitterion-buffered medium (e.g. HEPES, TEST, MOPS) if the incubator contains atmospheric air and a bicarbonate-based medium if the incubator contains an atmosphere of 5% CO2 (and if gas exchange is allowed) (11). Meanwhile, most commercially available sperm wash media contain zwitterions for pH buffering, although a certain level of bicarbonate is present as key capacitating agent for spermatozoa (65). Although these media are effective, there are concerns that zwitterion buffers may interfere with some important processes in different cell types and, consequently, have negative effects on gametes and embryos (66).

As far as we know, only one RCT (67) compared the PRs of sperm prepared with bicarbonate buffer and with HEPES buffer, in IUI with cryopreserved donor sperm (*n* = 324 cycles). This study reported significantly higher PRs when sperm was prepared using HEPES buffer. It has to be stated, however, that the effect might not be attributed to HEPES alone as two different culture media were used (HTF and HAM's F10). More RCTs are necessary on this subject, with stratification for normozoospermic and oligozoospermic men, and with temperature as important factor as the pH of buffers is temperature-dependent.

Temperature during centrifugation

It was suggested that the impact of the centrifugation temperature on sperm capacitation might mimic the impact of the storage temperature (68), as reported later in this review. In a group of 50 normozoospermic men, however, no significant difference was found in the level of DNA damage between samples centrifuged at controlled (testis or body temperature: 35 or 37°C, respectively) and non-controlled temperature (room temperature: ~25°C) (69). In another small group of normozoospermic men (*n* = 10), the percentage of motile sperm cells was higher after centrifugation at 34°C compared to centrifugation at room temperature (70). The samples centrifuged at 34°C were reported with a higher sperm yield, but only when they were also stored at this temperature before semen processing. Both studies provided no explanations for the temperature-dependent influences of semen centrifugation.

Only one RCT (*n* = 671) evaluated the impact of centrifugation temperature on IUI PRs. Included were couples with unexplained infertility and no differences were found in sperm parameters and IUI PRs between controlled and non-controlled centrifugation temperature (71). Based on this RCT and since non-controlled centrifugation is commonly used for reasons of ease, we conclude that further evaluation is not needed at this moment.

Temperature during storage

Usually, the storage of semen samples after preparation takes place at body temperature. Long-term storage (≥24 h) of spermatozoa at body or testis temperature, however, resulted in reduced motility and sperm quality (72,73). In general, reduced motility is observed both at room and body temperature in a time-dependent manner, but to a greater extent and more rapid at 37°C (74). Moreover, long-term storage at 37°C resulted in an increased incidence of large vacuoles in sperm nuclei (75). The positive impact of lower storage temperatures is explained by the switch of spermatozoa to a resting state, where better energy preservation might result in longer survival (73). This hypothesis is supported by the reported influence of storage temperature on some cellular mechanisms involved in sperm capacitation: a temporary blockage of capacitation-related events was present during storage at 20°C, but not at 37°C (68).

Clinical studies about the impact of storage temperature on PRs are missing. Furthermore, the above studies included small groups of men (*n* = 12–41) and did not specify the impact separately for fertile and infertile men. Further research is needed to evaluate the impact of storage temperature on IUI PRs. As literature is scarce, we can only recommend to avoid long-term storage at body temperature.

Table II. Main results of the randomized controlled trials comparing the IUI pregnancy rates between semen preparation techniques

Study	Included couples (n)	Cause of infertility	Mean post-wash TMSC (million)	Compared preparation techniques (PR per cycle)	Main results according to pregnancy rates
Grigoriou *et al.* (2005)*	52	Unexplained	20 20	- Sperm wash with PAF in medium (23%) - Swim-up (8%)	Sperm treated with PAF significant higher clinical pregnancy rate than direct swim-up technique
Posada *et al.* (2005)*	82 (121 cycles)	Not available	10.9 16.2	- Density gradient centrifugation (8%) - Swim-up (26%)	Significant increased clinical pregnancy rates in swim-up technique compared with density gradient centrifugation
Soliman and Goyal (2005)*	63	Not available	Not available	- Density gradient centrifugation (11%) - Wash-only (14%)	No superior technique
Xu *et al.* (2000)*	60	Male factor	41.4 24.3 32.3	- Wang tube sperm separation (45%) - Swim-up (15%) - Percoll^{R} density gradient centrifugation (20%)	Wang tube sperm separation methods significantly higher pregnancy rate than other two methods in oligoasthenoteratozoospermic men
Carrell *et al.* (1998)*	363 (898 cycles)	Female, male, unexplained	$\geq$ 20 $\geq$ 20 $\geq$ 20 $\geq$ 20 $\geq$ 20	- Sperm washing (7%) - Swim-up (13%) - Swim-down (6%) - Percoll^{R} density gradient centrifugation (13%) - Refrigeration/ heparin incubation (8%)	Swim-up and Percoll^{R} density gradient higher chance (not statistically significant) of pregnancy than other techniques Swim-down significantly lower pregnancy rate than swim-up and Percoll^{R} technique
Dodson *et al.* (1998)*	80 (153 cycles)	Female, male, unexplained	29 6 27	- Double centrifugation (15%) - Multiple-tube swim-up (14%) - Percoll^{R} density gradient centrifugation (20%)	No superior technique
Karamahmutoglu *et al.* (2014)	223 (338 cycles)	Unexplained, mild male	Not available	- SpermGrad^{R} density gradient centrifugation (17%) - Swim-up (7%)	Higher pregnancy rates in density gradient centrifugation compared to swim-up in couples with unexplained infertility

Study	Included couples (n)	Cause of infertility	Mean post-wash TMSC (million)	Compared preparation techniques (PR per cycle)	Main results according to pregnancy rates
Tsai *et al.* (2004)	121	Female ovulation dysfunction	Not available	- PureSperm^{R} density gradient centrifugation (13%) - Percoll^{R} density gradient centrifugation (14%)	No significant differences in pregnancy rate
Ragni *et al.* (1998)	121 (194 cycles)	Male, unexplained	8.5 7.6	- Swim-up (14%) - Swim-up with test yolk buffer (26%)	Test yolk buffer significantly increased pregnancy rate compared with standard swim-up
Zavos and Centola (1992)	148 (307 cycles)	Female, unexplained	29.4 26.7	- Double wash (10%) - SpermPrep^{R} filtration (21%)	SpermPrep^{R} filtration significantly higher clinical pregnancy rate per cycle than sperm wash

* Randomized controlled trials (RCTs) reported by Boomsma et al. (2007) comparing the effectiveness of gradient technique versus swim-up technique versus wash technique

TMSC= total motile sperm count, PR=pregnancy rate, PAF=platelet-activating factor

Timing of insemination

The timing of insemination comprises two variables: the detection/induction of ovulation and the time interval from this point to insemination. The WHO guideline provides no recommendations for one timing method over the other. According to the NICE, however, the use of basal body temperature charts does not reliably predict ovulation (4). In 2014, a review (76) included 18 RCTs about the effectiveness of different timing methods in natural and stimulated IUI cycles. When comparing hCG administration and LH surge as timing method for IUI, no differences in PRs were found, albeit the quality of evidence was low or very low. Additionally, double inseminations (e.g. at 24 and 48 h after ovulation induction) and the use of different types and dosages of hCG and GnRH-a resulted in no differences in IUI PRs (76,77).

Next to ovulation timing method, the timing of insemination can be discussed. The NICE guideline stated that insemination should be performed around ovulation (4). In literature, the comparison of different time intervals between ovulation induction and insemination showed no statistically significant differences in PRs (78-84). The majority of these studies compared time intervals between 24 and 48 h after ovulation induction. From a biological view, however, the insemination of sperm before ovulation might be favorable, i.e. at the time of ovulation induction (85,86). After intercourse, spermatozoa attach to the isthmus epithelium, where this binding keeps them viable and prevents capacitation (86). Moreover, this interaction results in de novo protein synthesis (87). Once ovulation occurs, a cascade of signals results in a hyperactivated sperm movement towards the oocyte (88). This ovulation-related timing mechanism is important, since an early start of capacitation resulted in apoptosis of the spermatozoa (89), while a late start of capacitation resulted in spermatozoa that were not equipped to recognize oocytes (88). Although the majority of these processes was found in animal studies with healthy subjects, it would be worthwhile to set up clinical studies to tests this theory in humans. Only one study compared PRs between injection simultaneously with administration and 34–36 h after hCG administration, but found no statistically significant differences (83).

Other treatment related factors might affect the correct moment of insemination. For example, embryos might be affected by premature luteinization, due to an early rise in progesterone at the end of the follicular phase in controlled ovarian hyperstimulated IUI cycles. This early rise of progesterone was observed in 22% of the cycles and led to reduced PRs from 23 to 8% (90). Also, human papillomavirus positivity was found to have a negative impact on IUI PRs (91).
Additional (multicenter) RCTs are recommended on all of these aspects.

IUI devices

The most important devices of influence on IUI results are laboratory and clinical disposables and media, like semen containers, wash media and catheters. Two possible impacts of these products can be distinguished: function and toxicity. With respect to function, the type of catheter and ultrasound guidance can be of influence. A soft tip catheter was found to cause less trauma to the endometrium compared to a hard tip catheter (92), but was not superior in PRs in a Cochrane review (93). Ultrasound guidance during insemination makes it possible to visualize the movement of the catheter inside the endometrial cavity and could so avoid endometrial trauma and uterine contractions (94). This ultrasound guidance did not result in higher PRs in comparative studies (95-97), it will only result in more complexity and higher costs.

For both laboratory and clinical equipment, cytotoxicity is a problem. Nijs and colleagues (98) state that toxicity can be caused by the composition of materials, the production process, the handling and packaging or the sterilization and transport processes. Using a human sperm survival assay (HSSA), these authors demonstrated that one type of sterile Pasteur pipette was related to a delayed manifestation of toxicity and that the inside lid of one type of sperm container caused an immediate negative impact on sperm motility. Others reported toxicity of certain ART products by use of a mouse embryo assay (MEA). The set up and validation of both assays is however poorly described, both biologically (99) and statistically (100). Also, pre-release clinical safety and effectivity tests of devices is missing in many cases and European legislation is unclear on this point (101). We conclude that additional well-described tests are needed before introduction of IUI and ART devices on the market.

Bed rest after IUI

The WHO guideline provides no recommendations for bed rest after IUI (11). The rationale for a positive impact of a short period of supine positioning after insemination is that the spermatozoa may reach the fallopian tube within only 10 min (102). Immediate mobilization might counteract this movement due to gravity (103).

Few RCTs evaluated the impact of 10–15 min of supine positioning on IUI PRs compared to direct mobilization. In two of these RCTs, with inclusion of 391 and 95 couples, supine positioning led to higher PRs (104,105). In disagreement with these findings, a recent RCT found no significant positive effect of bed rest after IUI. This study was performed in 479 couples with idiopathic or mild male subfertility (106). Possible explanations for these differences might be found in the indications for IUI and the number of treatments. For this moment, it is not possible to advise one policy over the other.

Levels of evidence

Table III gives a summary of the impact of different laboratory procedures on IUI success. Also, the corresponding levels of evidence (LOE) according to the NICE guideline (4) are shown. These LOEs are however more or less misleading, especially in the assignment of level 1a and 1b. In these cases, the included RCTs are most of the times characterized by the absence of standardized methods or small sample sizes, resulting in contradictive results. In general, it is remarkable that the procedures for IUI are characterized by a low level of evidence or insufficient literature even though IUI has been performed for decades. Even well-designed retrospective studies are missing, while these could be performed relatively simply and would lead to valuable information for efficiently setting up the more complex RCT's.

Discussion

The general conclusion of this review is that evidence is poor on most technical aspects of the IUI procedure. Different studies show contradictive results, mainly due to a low degree of standardization, low statistical power and inaccuracy in handling confounding factors. Nevertheless, some advise can be given to change the current guidelines.

We state that an EA period of up to 3 days is preferable to the 2–7 days described in the WHO manual. Furthermore, we advised avoiding long time intervals between semen production and processing. It is easier to perform centrifugation and storage at room temperature and this yields good results. Finally, zwitterion-buffered media might be preferred over bicarbonate-buffered media and IUI devices should be validated using HSSA.

Although only a part of these recommendations are really evidence-based, we think that they could be introduced for reasons of standardization, comfort (ease), quality control and costs. This does not mean that further research on these items is expendable. As literature is scarce, every new study can influence the recommendations. This was also the case for two items in this review: the time between sperm preparation and insemination and bed rest after insemination. In these cases, a recent retrospective study and RCT, respectively, reported different study results than the former literature, which resulted in a last-minute change of the recommendations.

Although RCTs can be preferred, multicenter retrospective studies could be informative as well in some cases, because these studies can give us the possibility to include the many variables that are present in the IUI process over the different clinics. In these studies, it is important that the participating clinics share the same

definitions on their data, e.g. for cycle number, pregnancy outcome and underlying diagnosis. Furthermore, a good registration of all technical variables is important. Next to clinical studies, for some variables, it could also be useful to perform biological experiments as alternative, like zona binding assays or measurement of DNA damage after different time intervals of incubation of prepared semen.

Based on the results of this review and in agreement with other IUI-related reviews (56,107), we emphasize the importance of standardization in IUI (study) protocols and guidelines. Here we meet another problem, since the readiness of clinics to follow existing guidelines is low (108,109), even when two different implementation strategies were used (110). An overview of guideline adherence of the laboratory stage of IUI is missing. We assume that this will be low also, because (older) studies ascribe limited willingness to follow guideline recommendations for processes related to IUI, like semen analysis (107,108,111-113) and to the vagueness and incompleteness of the recommendations, since supporting evidence is missing (111,114). We agree with earlier statements (115) that efforts should be made to improve guideline development and implementation by means of clinical results and economic consequences of IUI care.

This review indicates that further research on many IUI-related factors is necessary. We suggest to start with evaluating the current adherence to laboratory guidelines, e.g. by sending a questionnaire to fertility laboratories. This is also relevant with respect to semen analysis, as highlighted before (6); only a fraction of the laboratories is ISO 15189 accredited to the WHO standards for semen analysis (11). This may lead to wrong classification of semen samples and therefore disproportionate use of IVF and ICSI treatments.

Next, further research to update the current recommendations should include RCTs focusing on the impact on IUI success of wash medium buffers, storage temperature, timing of insemination (<24 h after ovulation induction) and bed rest. A first multicenter RCT could focus on two aspects of the sperm preparation method: gradient centrifugation compared to swim-up and bicarbonate compared to HEPES buffer. This study can be performed with enough power within a limited period of time. Whether aspects like EA and collection place should be studied in RCTs is point of discussion. In these cases, multicenter retrospective studies including patient and treatment characteristics (e.g. female age, cycle number, ovarian stimulation protocol) can be helpful instead.

With the results of these studies, guidelines can be updated and implementation strategies (e.g. educational materials or standardized training visits) can be drawn up. Subsequently, the effectiveness of the implementation strategy can be evaluated, both in pregnancy results and in costs.

Table III. Main conclusions of the included literature about the impact of different laboratory procedures on IUI pregnancy rates and summary of the recommendations and suggested next steps in research. Presented are the levels of evidence according to the NICE guideline (NICE, 2013) and the number of studies.

Variable	Level of evidence	Number of studies	Main conclusions in literature; reported procedure with highest PRs	Recommendations based on literature and WHO guideline	Next steps in research
Ejaculatory abstinence	3	2	EA up to 2 // 3 days	EA ≤ 3 days	Evaluation in RCTs, with stratification for oligo- and normozoospermic men
Collection place (clinic versus at home)	3#	2	Collection in the clinic // no difference	Either in the clinic or at home	Evaluation in RCTs, with stratification for oligo- and normozoospermic men
Time intervals	3#	4	Avoid short and long TIs // no impact	Sample delivered within 1 h after collection, avoid long TIs between semen collection-insemination and semen processing-insemination	In first instance in multi-center retrospective studies, separately for oligo- and normozoospermic men
Semen preparation technique	1a#	6 †@	No superior method	Method selection should be based on semen sample	Identification of methodologies with best IUI results in retrospective studies (e.g. number of layers, volume of medium)
Buffer of wash medium	1b	1	HEPES buffer better than bicarbonate buffer	Selection of the medium buffer should be based on used incubator	Additional evaluation in RCTs, with stratification for oligo-and normozoospermic men
Centrifugation temperature	1b	1	No difference between body // testis and room temperature	Non-controlled centrifugation temperature, for reasons of ease	None
Storage temperature	2 *	4	Storage at room temperature better than body temperature*	Avoid body temperature, especially during long-term storage	Evaluation of impact on PRs in RCTs, with stratification for oligo- and normozoospermic men
Method of timing IUI	1a	18 †	No superior method	No recommendable method	Evaluation in RCTs with standardized methods

Variable	Level of evidence	Number of studies	Main conclusions in literature; reported procedure with highest PRs	Recommendations based on literature and WHO guideline	Next steps in research
Time between ovulation and insemination	1b	7	No superior time interval	Insemination 24–48 h after ovulation induction	Evaluation in RCTs with standardized methods, including insemination <24 h after ovulation induction. With stratification for oligo-and normozoospermic men
IUI devices:	-	-	Some devices were reported as cytotoxic	Avoid the use of IUI devices that cause reprotoxicity	Development of well-described tests to identify safe and effective devices
Bed rest after IUI	1b	3	Bed rest of 10 // 15 min // no difference between bed rest and immediate mobilization	Either bed rest of 10–15 min or direct mobilization	Additional evaluation in RCTs, with stratification for oligo-and normozoospermic men

* Based on the impact on sperm parameters instead of pregnancy outcomes..

† Number of studies included in systematic review; # studies show contradictive results; @ number of studies in the meta analysis; // results of different studies.

EA = ejaculatory abstinence, TI = time interval, PR = pregnancy rate, RCT = randomized controlled trial.

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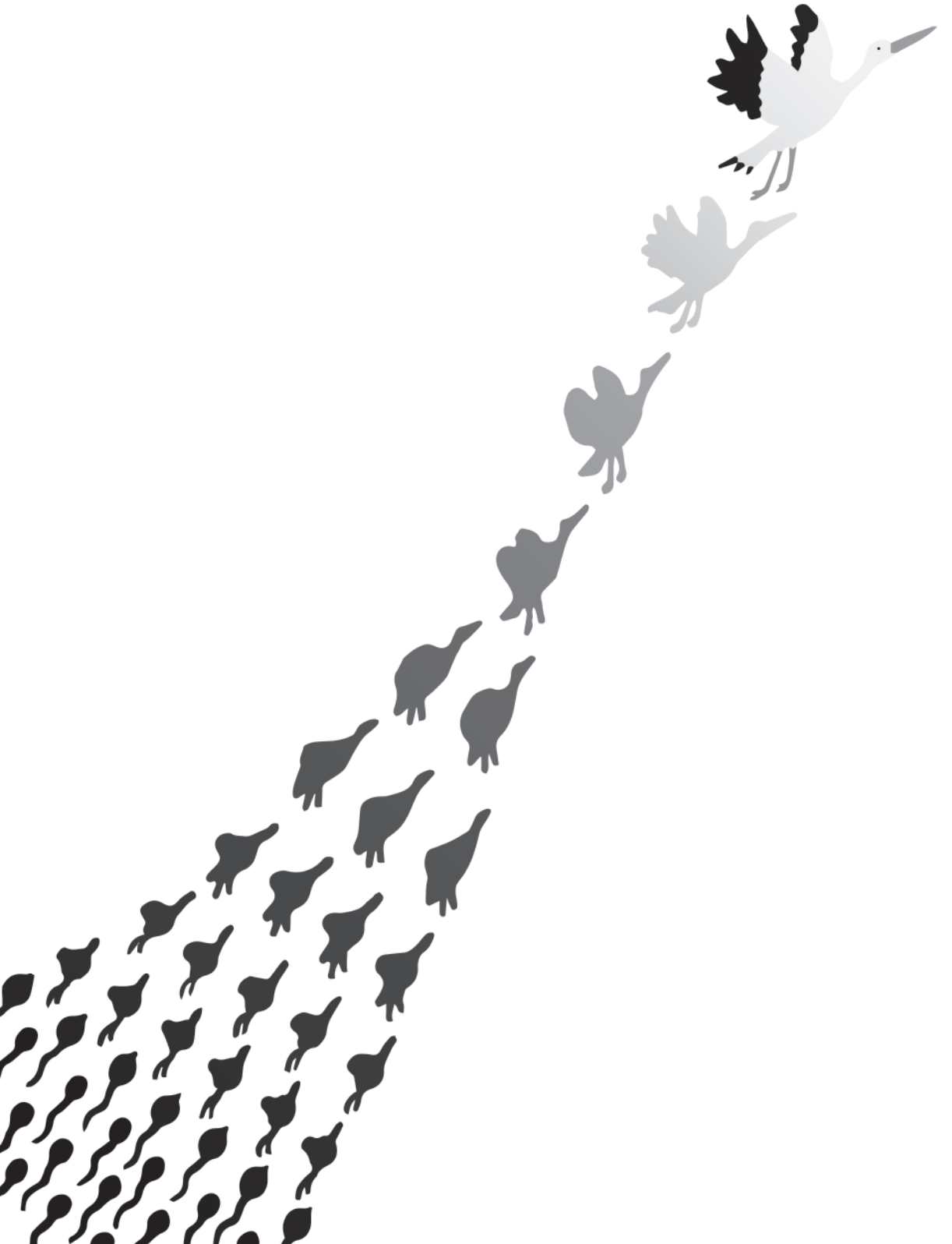

CHAPTER 3

Optimization of laboratory procedures for intrauterine insemination: survey of methods in relation to clinical outcome

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Abstract

Background: There is a wide practice variation of used methods and outcomes in IUI in fertility laboratories. Standardization of the IUI procedure is important for reducing inconsistency among laboratories in counselling infertile couples and in pregnancy results. The aim of the study was to evaluate the currently used laboratory procedures of IUI in Dutch fertility laboratories and their effect on IUI pregnancy results. Additionally, the methods for semen analysis (SA) were evaluated, as SA is related to IUI in terms of inseminated sperm number and IUI counselling.

Material and Methods: This questionnaire survey study was sent to laboratories participating in the Dutch external quality control program for semen analysis (SKML) and consisted of 46 questions concerning laboratory management, methods for semen analysis and IUI and clinical results. The results were analyzed using univariable and multivariable logistic regression models.

Results: A total of 52 laboratories (out of 99) provided information on used methodologies for SA or laboratory procedures of IUI and the organization of the laboratory. A wide variability was confirmed in used methods for both SA and IUI. Evaluation of pregnancy results obtained during 3 years (2013-2015) showed that specific used laboratory methods have a significant effect on the probability of becoming pregnant.

Discussion and conclusion: Important to remark is that in this survey study cycle specific data, including variables of the individual couples (age, stimulation protocol, etc) were not included and may have effects on the results. The reported results provide an overview of the current practice performance, however, the organization of fertility laboratories is changing rapidly. The use of standardized methods in IUI is important for optimizing the performance of care and improving pregnancy results. The knowledge on used procedures, however, is limited and further research on factors involving SA and the IUI procedure is necessary.

Introduction

Intrauterine insemination (IUI) is a commonly used procedure, conducted in many fertility clinics around the world (1,2). The results of the treatment are dependent on many factors, like female and male age, female factors, semen quality and treatment type (e.g. natural vs stimulated cycle, type of ovulation induction and timing of the insemination). The best indications for IUI are moderate male factor and unexplained infertility (3), with especially tubal factor infertility as contra-indication. Moreover, mild ovarian stimulation generally leads to better results than natural cycle IUI, thereby having a higher risk for multiple pregnancies (4,5). With respect to the effect of semen factors on IUI, studies report contradictive conclusions. Many authors report a minimum required motile sperm count (TMC) for effective IUI, however there is no consensus about the exact value of this minimum (6). Next to this minimum TMC, a maximum is reported in two studies (5,7). Also the impact of sperm morphology for successful IUI is under discussion. Although some groups report a certain impact of sperm morphology on the results of IUI, in a recent review it was concluded that it has a very poor clinical impact both in diagnostics and in prediction of pregnancy after ART (8). One possibility to explain the differences in outcome of these sperm counts is laid in the variation in practice of semen analysis (SA) by laboratories (6,8).

A same type of laboratory practice variation might be the origin of different pregnancy results after IUI. Despite the fact that there is a recommendation for both SA and sperm preparation for IUI (9), a recent literature review showed that the recommended methods for sperm preparation are characterized by a low level of evidence, insufficient literature and controversial results (10). On the other hand, from this literature review some tentative conclusions could be made for an optimal procedure.

An overview of guideline adherence by laboratories of the technical stage of IUI is missing in literature. Based on results of an earlier survey (not published), we expect a lot of variation, despite the fact that many laboratories introduced the ISO 15189 medical laboratory standard and besides an improved version of the WHO manual (9). As in the clinical part of IUI, where optimal guideline adherence was reported to have important economical benefits (11), this inter-laboratory variation is undesirable. On the other hand, coupling this variation in laboratory methods to IUI pregnancy rates, may provide more evidence with respect to the optimum procedure as extracted from literature (10).

Therefore the objective of this study is to survey the different laboratory IUI procedures, to associate these methods with IUI pregnancy results and to compare these outcomes with the conclusions of the literature review. As SA results are an important factor for IUI counseling (5,7,12), we also investigated the methods used

for SA. It is highlighted that the study is a laboratory survey and is not including individual patient data that can influence the pregnancy results of IUI. Moreover, the study was limited to the situation in Dutch fertility laboratories. As SA and IUI are performed on large scale in The Netherlands and the foreseen variation is high, a sanguine outcome can be expected, informative for other countries as well.

Materials and methods

About hundred Dutch fertility laboratories participating in the regular external quality control program for SA were invited for this questionnaire survey study. In the Netherlands, the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) is a non-profit organization that organizes the external quality control of most Dutch laboratories performing SA.

An invitation for the survey study was sent by e-mail to the laboratories, together with the weblink of the electronic questionnaire (Survey Monkey). One reminder was sent two weeks later. The responses were received in May and June, 2016.

Questionnaire development

The questionnaire was developed based on previous survey studies (13-17), our literature study (10) and input of an expert panel. The questionnaire consisted of 46 questions. Most questions were closed-ended, open-ended questions were used when the respondents should present quantitative data.

The questionnaire was constructed in Dutch and was designed to obtain information on the used methodologies of SA and IUI. In part A, the type of laboratory, quality management system and staff on the laboratory was questioned. Part B consisted of questions about the technical performance of SA (i.e. assessment of concentration, morphology and motility assessment) and the laboratory procedures of IUI (e.g. advised ejaculatory abstinence, semen collection place, temperature during centrifugation/storage). In part C, participants were requested to present their data on IUI ongoing pregnancy (fetal heart beat after 12 weeks of gestation) results in the period 2013-2015.

Statistical analyses

The results are presented as counts and percentages. The probability to become pregnant was modelled with logistic regression models, using the reported pregnancy results of 2013-2015. The dependent variable was pregnancy outcome (yes/no). The independent variables were semen collection place (at home or both in the clinic or at home), semen preparation technique (density gradient

centrifugation, washing, swim-up or swim-down), washing medium (HEPES or bicarbonate), temperature during storage (room temperature, body temperature or no storage), method of timing IUI (hCG administration, LH surge, ultrasound or a combination of these) and bed rest after insemination (direct mobilization or bed rest). The selection of independent variables was based on our literature review study (10). First, univariable logistic regression models were fitted. The statistically significant independent variables were included in a multivariable logistic regression analysis with forward selection based on the Wald test for selecting a set of variables predicting the probability to become pregnant (based on α=0.05). The crude odds ratios (ORs) with 95% confidence intervals (CIs) based on the univariable logistic regression model were estimated. The ORs with 95% CIs and the p-values of this final multivariable model are presented. The statistical analyses were performed using SPSS IBM Statistics 20.0 for Windows (Chicago, Illinois, USA).

Results

Ninety nine laboratories were invited for this study. A total 52 questionnaires was received on Survey Monkey (response rate of 52.5%). Of these respondents, 49 completed the SA part and 48 the IUI part of the questionnaire. Pregnancy results were reported by 35 (2014/2015) or 36 (2013) laboratories.

Organization of the participating clinics

Most of the respondents characterized their laboratory as a clinical chemistry laboratory (73.1%), others as specialized fertility/embryology (15.4%), clinical microbiology (5.8%) or clinical pathology (5.8%) laboratories. Of the clinical chemistry laboratories, two indicated that their fertility procedures were performed at a separated, specialized fertility setting. A total of 536 laboratory technicians were involved in performing SA and 549 in performing sperm processing for IUI. On average, the mean number of IUI treatments per technician is 40 a year. Of the total group of employees responsible for performing SA and/or semen processing for IUI, 9 (1.6%) were qualified clinical embryologists, 4 (0.7%) possessed another (post-) master's degree, 395 (68.6%) possessed a higher professional education, 166 (28.8%) intermediate vocational education and 2 (0.3%) had other degrees.

An interdisciplinary meeting with the department of gynaecology was organized once a year in 23.1% of the laboratories, twice a year in 21.2%, three or four times a year in 9.6% and more than four times a year in 20.9% of the laboratories. One-quarter of the participating laboratories indicated that such meeting did not take place at their clinic. Couples are treated following clinical IUI protocols with either a natural or stimulated cycle in most laboratories (93.8%). Couples in the remaining three clinics were offered only stimulated IUI cycles.

About 60% of the laboratories stated that they perform an internal quality control program, where most of these programs (74.2%) included a component specified to SA. All included laboratories were accredited according to the ISO 15189 or similar.

Semen analysis

A total of 49 laboratories responded to the subset of questions about SA methodologies, one reaction was incomplete. For SA, the WHO 2010 reference values were most frequently used (75.5% of the laboratories), followed by the WHO 1999 reference values (20.4%). One laboratory combined the reference values of the WHO 2010 and a Dutch directive (18).

The reported methodologies for SA are summarized in Table 1. Recommendations of the WHO guideline on SA procedures are followed by a limited number of laboratories: sperm concentration is determined using the improved Neubauer hemocytometer in 55.1% of the laboratories. With respect to sperm motility, about 40% of the laboratories use the WHO 1999 criteria (i.e. rapidly progressive, slowly progressive, non-progressive and immotile), which is recommended by the ESHRE Special Interest Group Andrology in their Basic Course on Semen Analysis (19). Moreover, participants of this course are instructed to assess sperm motility at body temperature. This is followed by 80% of the laboratories, in most cases by using a microscope stage heater (92.3%). Sperm morphology assessment seems to be considered of limited interest, because only a small majority of the laboratories (n=25) performed this test during routine SA. Only two laboratories assess sperm morphology using the Papanicolaou stain.

Table 1. Description of the used methods during semen analysis in the participating laboratories.

	Total group SA (n=49)	
Characteristic	**n**	**(%)**
Concentration		
Counting chamber		
Improved Neubauer	27	(55.1)
Makler	7	(14.3)
CellVision	5	(10.2)
Bürker Türk	4	(8.2)
Leja	4	(8.2)
SQA-V	2	(4.1)
Motility		
Guideline criteria		
WHO 2010	29	(59.2)
WHO 1999	19	(38.8)
Missing	1	(2.0)
Temperature during assessment		
Body temperature	39	(79.6)
Room temperature	9	(18.4)
Missing	1	(2.0)
Morphology (n=25)		
Staining method		
DiffQuik	12	(48.0)
Giemsa	4	(16.0)
Papanicolaou	2	(8.0)
Other	6	(24.0)
Missing	1	(4.0)

SA= semen analysis, WHO= World Health Organization

Laboratory procedures of IUI

Of the total group of respondents, 48 (92.3%) laboratories performed semen processing for IUI treatment. The majority of these, performed semen processing on six days (41.7%) or seven days (50.0%) a week, with no difference in clinical outcome. The used laboratory procedures of IUI are summarized in Table 2. The majority of the laboratories (72.9%) offered the couples the opportunity to collect semen both in the clinic and at home. In these clinics, semen collection usually took place at home in 25 laboratories (71.4%), at the clinic in 25.7% and only one laboratory stated that collection took place in both options at a comparable frequency. Moreover, 31.3% of the participating laboratories advised an ejaculatory abstinence period of 2-7 days before semen collection (WHO recommendation for SA). Other advised ejaculatory abstinence periods were 2 days (18.8%), 3 days (18.8%), 2-5 days (6.3%) or 5-7 days (6.3%).

Most of the respondents (89.6%) reported routinely using density gradient centrifugation as semen preparation technique, followed by conventional washing (6.3%). The used density gradients were PureSperm^{R} (Nidacon, Gothenberg,

Sweden) in 23.3% of the laboratories, SilSelect^{R} (FertiPro, Beernem, Belgium) in 65.1% of the laboratories and other, less common gradients (i.e. SpermGrad^{R}, SpermPrep^{R}, SupraSperm^{R} and SpermTec^{R}) in 11.6% of the laboratories. The density gradients were used in a single layer (30.2%) or a double layer (69.8%).

Table 2. Description of the used laboratory procedures of IUI in the participating laboratories.

	Total group IUI (n= 48)	
Characteristic	**n**	**(%)**
Semen collection		
Semen collection place		
At the clinic	1	(2.1)
At home	12	(25.0)
Both	35	(72.9)
Recommended ejaculatory abstinence		
2-7 days	15	(31.3)
2 days	9	(18.8)
3 days	9	(18.8)
2-5 days	3	(6.3)
5-7 days	3	(6.3)
Other	9	(18.8)
Semen processing		
Semen preparation technique		
Density gradient centrifugation	43	(89.6)
Washing	3	(6.3)
Swim-up	1	(2.1)
Swim-down	1	(2.1)
Temperature during centrifugation		
Room temperature	48	(100.0)
Body temperature	0	(0.0)
Washing medium		
HEPES	37	(77.1)
Bicarbonate	8	(16.7)
Missing	3	(6.3)
Temperature during storage		
Room temperature	30	(62.5)
Body temperature	11	(22.9)
No storage	4	(8.3)
Missing	3	(6.3)
Insemination		
Method of timing IUI		
hCG administration	16	(33.3)
LH surge	3	(6.3)
Ultrasound	3	(6.3)
Combination	22	(45.8)
Missing	4	(8.3)
Bed rest after insemination		
Direct mobilization	9	(18.8)
Bed rest	36	(75.0)
Missing	3	(6.3)

All respondents centrifuged the semen samples at room temperature. HEPES buffered wash media (zwitterion-buffered) were commonly used and, pending insemination, the samples were stored at room temperature in 62.5% of the laboratories. Most laboratories reported a combination of methods for timing the moment of IUI (i.e. hCG administration, LH surge or ultrasound). The reported time intervals between ovulation induction and insemination ranged between 8 and 48 hours (median 36 hours). After insemination, patients were asked to have bed rest for 10 minutes in one-quarter of the clinics, 10-15 minutes in 5.6% and 15 minutes in 55.6% of the clinics, where others reported less common durations (i.e. 5, 20 or 30 minutes). In 18.8% of the clinics no bed rest was advised.

Pregnancy results

About 35 laboratories presented their data on the number of performed SA, IUI cycles and the pregnancy rates in 2013-2015. Table 3 summarizes these data, together with the calculated pregnancy rates per cycle. Overall, a total of 42,071 SA were performed in these clinics in the period of 2013-2015. Moreover, a total of 3,734 ongoing pregnancies were reported in 42,613 IUI cycles. Subsequently, the mean pregnancy rates per cycle were 9.4%, 9.6% and 9.6%, respectively, in 2013, 2014 and 2015. Regarding the organization of the participating laboratories, specialized laboratories (i.e. fertility/embryology) performed on overage more SAs (1,171 vs. 346) and IUI treatments (763 vs. 286) than other laboratories (i.e. clinical chemistry, microbiology and pathology), while pregnancy rates were comparable (9.4% in specialized laboratories vs. 9.5% in others). This might indicate that both types of laboratories use the same indications and/or have the same variation in methods for IUI. Pregnancy rates were significantly lower in clinics performing IUI 5 days a week, while these were comparable in clinics with either a 6 or 7 days service.

Table 3. Total number of performed semen analyses and IUI cycles over the last years, together with the IUI ongoing pregnancy results.

	2013		2014		2015	
Number of laboratories	**Number of laboratories**	**N or %**	**Number of laboratories**	**N or %**	**Number of laboratories**	**N or %**
Semen analyses	28	14,394	30	13,924	30	13,753
IUI cycles	38	14,407	38	13,639	38	14,567
Ongoing pregnancies	36	1,312	35	1,189	35	1,233
PR per cycle (mean of labs)*	36	9.4%	35	9.6%	35	9.6%
PR per cycle (overall) †	36	9,1%	35	8,7%	35	8,4%

PR= pregnancy rate, * Sum of PRs per laboratory/number of laboratories, † Total number of pregnancies/ total number of cycles.

In Table 4, the crude ORs from the univariable and the multivariable logistic regression models are presented. The probability to become pregnant was positively influenced by the use of a HEPES buffered washing medium compared to bicarbonate buffer (p=0.02) and by storage at room temperature compared to storage at body temperature (p=0.00). Higher pregnancy rates were also obtained in clinics that give the possibility to collect semen at home or in the clinic than in clinics that advice semen collection at home only (OR=0.71, 95%CI (0.59-0.87)). Wash-only had a negative effect compared to density gradient centrifugation (OR=0.77, 95%CI (0.64-0.94)). Furthermore, the method of timing IUI was an independent variable, with a lower probability in the group using hCG administration (OR=0.80, 95%CI (0.73-0.87)). This model was based on data from 33,233 reported cycles in the participating laboratories; in the other cycles were one or more variables missing.

Discussion with conclusions

As expected, this questionnaire survey study shows a wide variability of used methods on fertility laboratories in the Netherlands, especially for SA methods. With respect to IUI, some of the laboratory variables have a significant impact on reported pregnancy rates.

One possible explanation for the variation in methods may be found in the fact that the presented data give us an overview of the situation in 2016. Some of the respondents replied that the used methods were recently changed or that changes are planned in the near future due to re-organizations. This will especially bias the impact of different laboratory procedures on IUI outcome (Table 4), since the reported methods were not always used during the total study period 2013-2015. The number of laboratories performing SA in the Netherlands is changing, mainly as a result of fusions of laboratories. This may lead to different points of view within the fused group of professionals.

In general, the measured variation is both alarming and interesting. Alarming, because by absence of reference material, a recommended and fully validated method is all we have. Using other, non-validated methods adds to the uncertainty associated with the quality of analysis and makes it inappropriate for laboratories to use WHO reference values. So, the situation could theoretically lead to inadequate patient selection for the different treatments and so possibly to lower results of these treatments. We have to be careful in this, because from this survey it is not clear whether or not alternative methods are validated against the WHO recommendations. Moreover, it should be mentioned that SA not only contributes to predicting pregnancy rates, but is also valuable in a wider andrological perspective: a poor result from SA should be a starting

point for further evaluation of the man's health, e.g. by testicular ultrasound or endocrine examination. The interesting part of the variation is laid in the fact that all responding laboratories were accredited according to ISO 15189 (or CCKL, a national erstwhile standard similar to ISO) but that this is not a guarantee that they follow the WHO recommendations. A possible explanation may be that accrediting bodies do not refer to these recommendations as they are no official standard (9,20). A way to overcome this problem may be to write an ISO standard for SA and/or by implementing a check-list on SA by major scientific journals (Bjorndahl, 2016). On the other hand, these "top-down" strategies might not work as there apparently is a kind of resistance to follow the present recommendations.

Table 4. The odds ratios of the laboratory procedures for the probability to become pregnant after an IUI treatment, based on the reported ongoing pregnancy rates

		Univariable			Multivariable *		
Characteristic	**n**	**OR**	**(95% CI)**	**p-value**	**OR**	**(95% CI)**	**p-value**
Semen collection place							
At the clinic	0	-					
At home	5,381	0.89	(0.80-0.98)	0.02	0.71	(0.59-0.87)	0.00
Both	33,926	1.00	(Ref)		1.00		
Semen preparation technique							
Density gradient centrifugation	34,395	1.00	(Ref)		1.00	(Ref)	
Washing	2,623	0.72	(0.61-0.84)	0.00	0.77	(0.64-0.94)	0.01
Swim-up	1,020	1.13	(0.92-1.39)	0.24	1.27	(1.03-1.57)	0.03
Swim-down	1,269	1.05	(0.87-1.27)	0.59	-		
Washing medium							
HEPES	30,838	1.00	(Ref)		x		
Bicarbonate	6,043	0.89	(0.80-0.98)	0.02			
Temperature during storage							
Room temperature	29,205	1.00	(Ref)		x		
Body temperature	7,128	0.86	(0.79-0.94)	0.00			
No storage	1,522	0.98	(0.82-1.17)	0.84			
Method of timing IUI							
hCG administration	14,651	0.85	(0.79-0.91)	0.00	0.80	(0.73-0.87)	0.00
LH surge	2,094	0.98	(0.84-1.14)	0.80	0.94	(0.81-1.10)	0.45
Ultrasound	738	0.63	(0.47-0.85)	0.00	0.85	(0.60-1.21)	0.37
Combination	18,868	1.00	(Ref)		1.00	(Ref)	
Bed rest after insemination							
Direct mobilization	14,305	1.00	(Ref)		x		
Bed rest	23,550	0.97	(0.90-1.04)	0.37			

OR= odds ratio, CI= confidence interval, Ref= reference, - = no data to include, x= not included in the model

* AUC of the model was 0.54 (95%CI (0.53-0.55)), Nagelkerke $R^2 < 0.001$.

This resistance arose from previous versions of the WHO manual (21,22) that were judged as incomplete and their introduction did not result in the use of standardized materials and methods among fertility laboratories for SA (13-17) and IUI (14,23). The most recent WHO manual can also be discussed, especially with respect to

agreeability of the procedures. It can be expected that there is more willingness to implement recommendations with a higher level of evidence and/or simplicity. A good example of this is the examination of sperm morphology. In The Netherlands, this test is out of interest because gynaecologists mainly use the validated Hunault model as predictive tool for pregnancy (24,25). In this model, sperm morphology is not included as a parameter of relevance, which may lead to less willingness to perform the test or to perform it accurately. The questionnaire results confirm this hypothesis: only half of the laboratories perform sperm morphology assessment of which only two use the Papanicolaou stain, as described by the WHO. A similar result was found in France (26). The risk of not performing sperm morphology assessment is complex to speculate about, as on the one hand one might miss specific severe abnormalities which lead to infertility (e.g. globozoospermia, 100% short motile tails, 100% severe ERC and 100% stress induced elongated sperm heads) and, on the other hand, a poor performance may lead to unsuitability of the algorithm used for patient selection. With respect to the first point, The dilemma is that the incidence of these specific abnormalities is very low and therefore it will be difficult to train and instruct standard laboratories on these items.

Next to agreeability, also interpretation of the WHO recommendations can be a source of variation. For example, the abstinence time for SA as advised by WHO (2-7 days) is used by many laboratories also for IUI (table 2). The WHO does however not give recommendations on this aspect for IUI and shorter intervals are probably more effective for this treatment (27-29). The same is true for semen collection at home. As the WHO manual states that analysis should begin within one hour after ejaculation, this may be interpreted that there is some freedom in the sampling step. Some accreditation programs might see this as a high risk for loss of process control.

Next to the laboratory aspects, IUI guideline adherence in clinical studies was reported as far from optimal with a large variability between different hospitals (30). Even the use of a professional-directed strategy and a multi-faceted patient- and professional-oriented strategy did not improve the implementation of a set of clinical guideline recommendations (31). Like shortcomings in the performance of SA, this could lead to a less careful inclusion of patients, thereby decreasing the effectiveness of the treatment. So, with respect to IUI guidelines, implementation on the laboratory and clinic face similar problems. Moreover, this questionnaire study reported a limited number of team meetings with both clinic and laboratory professionals. We think that these interdisciplinary meetings, where clinical results should be systematically reviewed, are an important condition for best practice and quality improvement. A guideline combining both laboratory and clinical procedures could be the solution to overcome these problems. A first effort was made by reviewing the essential steps for the laboratory stage of IUI, including the pre and post laboratory processes (10), thereby overlapping parts of the clinical process.

From our literature review (10), two conclusions could be drawn. First, most of the laboratory steps were characterized by a low level of evidence and, second, if evidence is available, guidelines do not always recommend this best practice. Surprisingly, when comparing process variables from this questionnaire with the results of the literature review (10), it was found that there was an accordance of ≥75% on variables with the highest level of evidence (level 1 evidence (32)). On the other hand, however, the review study reported no superior method for the variables which had significant influences on the probability to become pregnant in this study. So, it seems that this simple questionnaire study can further argument the results from literature. This is interesting, however caution is needed when interpreting some of these results, since these were based on a small group of fertility laboratories. For example, patients treated in clinics performing their IUI procedures during 5 days a week had a statistically significant lower probability to become pregnant (OR= 0.77, 95%CI (0.67-0.89)) compared to clinics with a 7 days week schedule. The first group, however, consists of only 3 laboratories. Another important remark is that this study included clinic specific data, while cycle specific data, including variables of the individual couples (age, stimulation protocol, etc), would be more accurate. It may seem, for instance, that semen collection at home was less successful than for clinics allowing both home and clinic collection. However, this association is not as per definition causal as patients were not allocated randomly to either situation. Furthermore, the predictive power (area under the ROC-curve) of the final model was low (AUC=0.54, 95%CI (0.53-0.55)).

Altogether, the different laboratory procedures for both SA and IUI need more supporting evidence related to “best practice”, resulting in convincing guidelines which should be combined with clinical guidelines. A first step to reach this goal can be found in the most recent validation of the Hunault model in which, next to sperm motility, also sperm morphology and volume are included (33). In fact this is a recognition of previous studies in which all semen parameters were found to be relevant for predicting fertility (34-36) and this could be a motivation for laboratories to follow the guidelines and to improve their procedures. Furthermore, to constantly improve the guidelines, additional research is essential. The next step to evaluate the influences of laboratory procedures on pregnancy results is the use of well-designed, multicenter controlled (retrospective or prospective) trials using couple-specific data. Thereafter, an effort can be made to optimize care by improving the implementation of best practice in the IUI laboratory.

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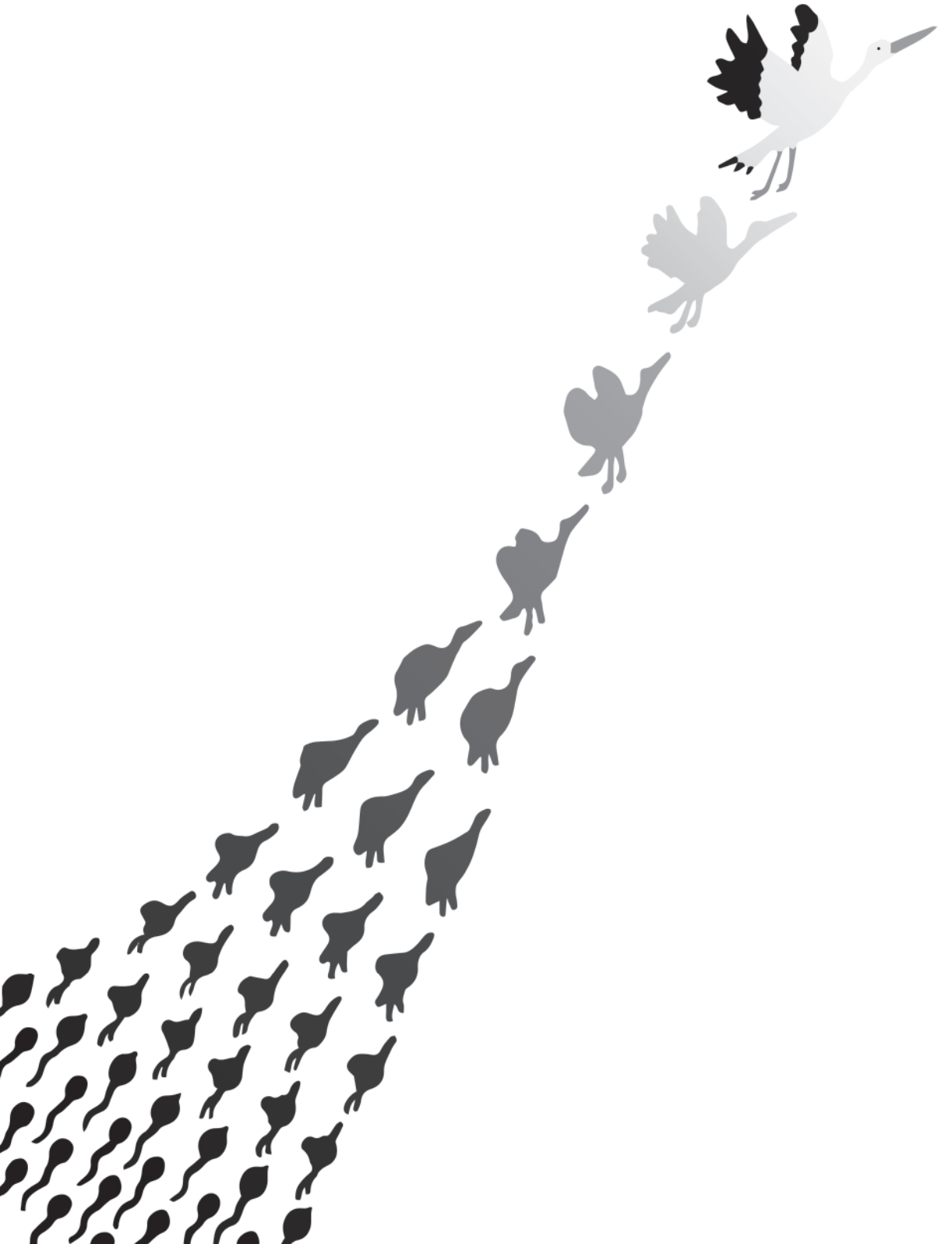

CHAPTER 4

Predictive value of sperm morphology and progressively motile sperm count for pregnancy outcomes in intrauterine insemination

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Abstract

Objective: To investigate the value of sperm parameters to predict an ongoing pregnancy outcome in couples treated with intrauterine insemination (IUI), during a methodologically stable period of time.

Design: Retrospective, observational study with logistic regression analyses.
Setting: University hospital.

Patient(s): A total of 1,166 couples visiting the fertility laboratory for their first IUI episode, including 4,251 IUI cycles.

Intervention(s): none

Main Outcome Measure(s): Sperm morphology, total progressively motile sperm count (TPMSC), and number of inseminated progressively motile spermatozoa (NIPMS); odds ratios (ORs) of the sperm parameters after the first IUI cycle and the first finished IUI episode; discriminatory accuracy of the multivariable model.
Results: None of the sperm parameters was of predictive value for pregnancy after the first IUI cycle. In the first finished IUI episode, a positive relationship was found for ≤4% of morphologically normal spermatozoa (OR 1.39) and a moderate NIPMS (5–10 million; OR 1.73). Low NIPMS showed a negative relation (%1 million; OR 0.42). The TPMSC had no predictive value. The multivariable model (i.e., sperm morphology, NIPMS, female age, male age, and the number of cycles in the episode) had a moderate discriminatory accuracy (area under the curve 0.73).

Conclusion: Intrauterine insemination is especially relevant for couples with moderate male factor infertility (sperm morphology ≤4%, NIPMS 5–10 million). In the multivariable model, however, the predictive power of these sperm parameters is rather low.

Introduction

In a previous study, we evaluated the prognostic value of sperm morphology to predict pregnancy outcomes in couples treated with in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (1). Our research filled a gap in the literature on the subject, which dated from the period before the introduction of ICSI in the 1990s and thus needed an update. Over the past decades, the reported percentages of morphologically normal spermatozoa has decreased with the introduction of stricter criteria and the tendency toward lower reference values (2). This is a disturbing factor in the use of sperm morphology as a prognostic factor for the probability of achieving a pregnancy. The strength of our study (1) was the selection of a stable period of time (i.e., 2004 to 2011) with respect to the methodology of sperm morphology assessment. In contrast to older studies, as reviewed by Coetzee et al. (3), we concluded that sperm morphology has no prognostic value in individual in vitro fertilization (IVF) and ICSI patients.

However, we can hypothesize that the role of sperm factors in intrauterine insemination (IUI) is different from their role in IVF and ICSI. Literature reviews reveal an ongoing debate on the value of sperm parameters as predictors of IUI outcome (4-6). More specifically, conflicting results have been reported for the influence of sperm morphology assessment using strict criteria on pregnancy outcomes with IUI (7-16). These studies are characterized by a lack of standardization, so repeating our previous study with couples treated with IUI is valuable.

Besides sperm morphology, there is disagreement about the predictive value of the total progressively motile sperm count (TPMSC) to predict IUI outcomes (6). In their review, Ombelet et al. (4) stated that the TPMSC has a substantial discriminative value. Others, however, have concluded that the TPMSC has poor sensitivity for selecting the couples most likely to conceive with IUI, but high specificity for identifying the couples unlikely to conceive with IUI (6,17,18). The required number of inseminated progressively motile spermatozoa (NIPMS) is under discussion as well, although in general a minimum of 5 million spermatozoa is stated as accurate (2).

We investigated the value of sperm parameters to predict ongoing pregnancy outcomes in couples treated with IUI during a methodologically stable period of time. The sperm parameters studied were sperm morphology and TPMSC, both assessed during fertility workup, and NIPMS assessed at the time of IUI. Additionally, the predictive power of these parameters for the probability of achieving a pregnancy is examined in conjunction with other known predictors.

Materials and methods

Study population

In this retrospective, observational study, anonymized data sets were included of all couples who visited the fertility laboratory of the Radboud University Medical Centre Nijmegen for confirmed, finished IUI episode between January 1, 2004, and June 30, 2013. A finished episode is defined as a sequence of treatment cycles that ends when a cycle results in a pregnancy or when IUI treatment is stopped. Records were excluded when data on pregnancy outcome or sperm parameters were missing. In cases where multiple assessments of morphology and/or TPMSC were performed, the data from the most recent fertility workup were used. The ethics review board of the Radboud University Medical Center Nijmegen provided approval for this study.

Study period

The study period was based on the stability of methods for semen analysis and semen preparation. In our previous study (1), we established this period between the years 2004 and 2011. Our methods did not change since 2011; the period for the present study was extended to June 30, 2013.

Semen analysis

Sperm samples were collected preferably after 2 to 3 days of ejaculatory abstinence and were delivered to the laboratory within 1 hour. Semen analysis was performed as described in our previous study (1). Briefly, the volume was determined by aspirating the ejaculate with a scaled pipette, the sperm concentration was determined by counting in a Makler chamber, and the fraction of progressively motile spermatozoa was determined in a 20-μm deep wet preparation. For the sperm morphology assessment, a small drop of semen was mixed with an equal amount of aniline blue/eosin solution, which consisted of 2 g of eosin yellow and 25 g of aniline blue (VWR) in 100 mL of phosphate-buffered saline (Gibco-Invitrogen) and 1 mL of ethanol. The mix was spread on a microscopic slide and flame fixed. A total of 200 spermatozoa per slide were evaluated according to the current World Health Organization (WHO) criteria at an original magnification of ×1,000 (19, 20). From this sperm assessment during the fertility workup, the percentage of morphologically normal spermatozoa and the TPMSC were calculated.

Intrauterine insemination

We performed IUI in natural cycles or in cycles with mild ovarian stimulation. The semen preparation was performed using a one step (80%) PureSperm (Nidacon) gradient after dilution of the semen with 5-mL of wash medium (human tubal

fluid medium; Gynotec) supplemented with 10% albumin (GPO; Sanquin). After centrifugation (500 × g), the semen was washed with wash medium, and the NIPMS was assessed in the samples used for insemination. The sperm variables were determined as described earlier, and the NIPMS was calculated by multiplying the volume by sperm concentration and the fraction of progressively motile sperm of the prepared semen. Approximately 2 weeks after insemination, a pregnancy test was performed; 8 to 10 weeks later, an ultrasound examination was used to confirm an ongoing pregnancy.

Assessment of variables

The main variables of interest in the predictive model were the percentage morphologically normal spermatozoa and the TPMSC. Both diagnostic sperm parameters were assessed routinely during the fertility workup. Additionally, the NIPMS was analyzed to investigate whether information about the inseminated sample improved the predictive value of the model. Note that this sample was not the same as the sample used during the fertility workup. Besides sperm parameters, we evaluated the effects of underlying female factor etiology (i.e., ovulatory dysfunction, cervical factor, endometriosis, or tubal/uterine abnormalities), female age, male age, and the number of IUI cycles. Moreover, we studied the number of IUI cycles until an ongoing pregnancy was established within the first finished IUI episode. The outcome variables were ongoing pregnancy after the first IUI cycle and ongoing pregnancy in the first finished IUI episode of a couple.

Statistical methods

The baseline population characteristics are presented as a median and range or as a count and percentage. Based on previous reviews (4, 5), we categorized the percentage morphologically normal spermatozoa into two groups: ≤4% and >4%. The TPMSC was categorized into four groups (i.e., ≤20 million, 20–50 million, 50–100 million, and >100 million), as was the NIPMS (i.e., ≤1 million, 1–5 million, 5–10 million, and >10 million). The NIPMS was assessed at each IUI cycle, so the mean NIPMS of the first finished IUI episode of the couple was calculated before categorization.

The different cycles within a couple are not independent of each other, so ignoring this dependency during data analysis might result in incorrect conclusions (21). For this reason, we studied the probability of becoming pregnant in both the first IUI cycle and the first finished IUI episode. In the first instance, univariable logistic regression analysis was used to determine the influence of sperm morphology and TPMSC. The dependent variable was pregnancy outcome after the first IUI cycle and in the first finished IUI episode, respectively. The independent variable was sperm morphology (≤4% or >4%) or TPMSC (≤20 million, 20–50 million,

50–100 million, or >100 million). Similar logistic regression was used to study the other IUI-associated variables. The crude odds ratios (ORs) with 95% confidence interval (CI) were calculated.

Subsequently, multivariable logistic regression with a forward selection procedure was used to identify those variables that independently predict the probability of becoming pregnant after the first IUI cycle and in the first finished IUI episode, respectively. The adjusted ORs with the appropriate 95% CI and the discriminatory accuracy (i.e., the area under the receiver operating characteristic curve [AUC]) of the final model are presented. All statistical analyses were performed using SPSS IBM Statistics 20.0 for Windows.The baseline population characteristics are presented with their median and range or with their count and percentage. Based on previous reviews (4, 5), the percentage morphologically normal spermatozoa was categorized into two groups (i.e. ≤4% and >4%). The TPMSC was categorized into four groups (i.e. ≤20 million, 20-50 million, 50-100 million and >100 million), as well as the NIPMS (i.e. ≤1 million, 1-5 million, 5-10 million and >10 million). The NIPMS was assessed at each IUI cycle and, therefore, the mean NIPMS of the first finished IUI episode of the couple was calculated, prior to categorization.

Results

In Table 1, the baseline characteristics of the total population and of the two groups by percentage of morphologically normal spermatozoa are shown. In total, 1,166 couples with 4,251 IUI cycles were included in this study. Of these, 112 couples (9.6%) became pregnant after their first IUI cycle, and 329 (28.2%) became pregnant in their first finished IUI episode. The percentage of morphologically normal spermatozoa was ≤4% in 444 couples and >4% in 629 couples. As expected, the higher levels of TPMSC (i.e., >100 million) and NIPMS (i.e., >10 million) as well as the number of couples with underlying female factor infertility were more frequently observed in the group with >4% normal sperm morphology than in the group with ≤4% normal sperm morphology. The ongoing pregnancy rates in the first IUI cycle were comparable in these two groups (10.8% and 9.2%, respectively). Surprisingly, the group with ≤4% normal sperm morphology had the highest ongoing pregnancy rate after the first finished IUI episode (32.9% vs. 26.1%).

The descriptive statistics of the four TPMSC groups are summarized in Table 2. With respect to the ongoing pregnancy rate, the four groups showed comparable results in both the first IUI cycle (9.4%, 10.0%, 8.5%, and 10.8%, respectively) and the first finished IUI episode (29.3%, 31.5%, 25.6%, and 28.8%, respectively). The increase in TPMSC was related to the increased percentage of couples with underlying female factor infertility (23.9% toward 38.1%) and with unexplained

infertility (7.4% toward 48.3%). The percentage of couples with NIPMS >10 million increased with higher values of the TPMSC (15.2% toward 87.7%).

First IUI cycle

In Table 3, the ORs are presented of the probability to become pregnant after the first IUI cycle and in the first finished IUI episode, respectively, using univariable logistic regression. Both the percentage of morphologically normal spermatozoa and the TPMSC were not statistically significant when related to the probability to become pregnant after the first cycle. It turned out that female age and male age were the only variables that were statistically significantly related to the probability of becoming pregnant after the first IUI cycle. A lower probability to become pregnant was related to both increased female age (OR 0.93; 95% CI, 0.89–0.98) and increased male age (OR 0.96; 95% CI, 0.92–1.00). Only female age was found to be predictive in multivariable regression analyses (Table 4). The predictive power of this model, however, was rather low (AUC 0.58; 95% CI, 0.53–0.64).

First finished IUI episode

The probability of becoming pregnant in the first finished IUI episode was higher in the group with normal sperm morphology of ≤4% as compared with the group with >4% morphologically normal spermatozoa (OR 1.39; 95% CI, 1.06–1.81) (Table 3). The group with a NIPMS ≤1 million showed a statistically significant lower probability (OR 0.42; 95% CI, 0.23–0.76) and the group with a NIPMS between 5 and 10 million a higher probability of becoming pregnant (OR 1.73; 95% CI, 1.21–2.46) compared with the group with a NIPMS >10 million. Sperm morphology and NIPMS were also independent predictors of the probability to become pregnant in the multivariable analysis (Table 4).

The probability of becoming pregnant in the first finished IUI episode was negatively influenced by higher female age (OR 0.92; 95% CI, 0.88–0.96), higher male age (OR 0.97; 95% CI, 0.94–1.00), and an increasing number of cycles within the episode (OR 0.73; 95% CI, 0.68–0.78). This model was based on data from 1,072 couples; the other couples were excluded because one or more variables were missing. The predictive power (AUC) was 0.73 (95% CI, 0.70–0.77).

Discussion

This study reveals that couples with a lower percentage of morphologically normal spermatozoa (i.e., ≤4%) and a moderate NIPMS (i.e., 5–10 million) had the highest probability of becoming pregnant in the first finished IUI episode. In couples with a NIPMS of ≤1 million this probability was lower. The TPMSC had no predictive value in this perspective. In general, the predictive power of the semen parameters was limited, and it seems that especially female factors were critical for predicting the probability of becoming pregnant in individual couples.

Table 1. Descriptive statistics of the first finished IUI episode of couples, assorted by the group of percentage morphologically normal spermatozoa

	Total group (n=1,166)		**Percentage morphologically normal spermatozoa**			
			≤4% (n=444)		**>4% (n=629)**	
	Median (range)/n (%)		**Median (range)/n (%)**		**Median (range)/n (%)**	
Female age	33	(21-41)	32	(21-41)	33	(21-41)
Male age	35	(23-67)	35	(24-57)	35	(23-67)
Etiology factor						
female factor	386	(33.1)	102	(23.0)	249	(39.6)
unexplained	373	(32.0)	124	(27.9)	214	(34.0)
TPMSC						
≤20 million	297	(25.5)	197	(44.4)	100	(15.9)
20-50 million	219	(18.8)	109	(24.5)	110	(17.5)
50-100 million	223	(19.1)	83	(18.7)	140	(22.3)
>100 million	333	(28.6)	55	(12.4)	278	(44.2)
Unknown	94	(8.1)	0	(0.0)	1	(0.2)
NIPMS						
≤1 million	103	(8.8)	73	(16.4)	24	(3.8)
<5 million	236	(20.2)	142	(32.0)	80	(12.7)
5-10 million	171	(14.7)	78	(17.6)	82	(13.0)
>10 million	655	(56.2)	150	(33.8)	443	(70.4)
Unknown	1	(0.1)	1	(0.2)	0	(0.0)
OP first cycle	112	(9.6)	48	(10.8)	58	(9.2)
OP first finished episode	329	(28.2)	146	(32.9)	164	(26.1)
Nr of cycles in episode	3	(1-21)	3	(1-14)	3	(1-21)
Nr of cycles till pregnancy *	2	(1-11)	2	(1-11)	2	(1-11)

*based on the number of cycles with an ongoing pregnancy in the first finished IUI episode

TPMSC= total progressively motile sperm count, NIPMS= number of inseminated progressively motile spermatozoa,

OP= ongoing pregnancy. TPMSC and NIPMS are measured as volume × concentration × progressively motile spermatozoa

Table 2. Descriptive statistics of the first finished IUI episode of couples, assorted by the group of total progressively motile sperm count (TPMSC)

	Total progressively motile sperm count							
	≤20 million (n=297) Median (range)/n (%)		20-50 million (n=219) Median (range)/n (%)		50-100 million (n=223) Median (range)/n (%)		>100 million (n=333) Median (range)/n (%)	
Female age	32	(21-41)	33	(21-41)	33	(22-41)	34	(22-41)
Male age	35	(23-67)	35	(25-56)	35	(24-56)	36	(24-53)
Etiology factor								
female factor	71	(23.9)	73	(33.3)	80	(35.9)	127	(38.1)
unexplained	22	(7.4)	60	(27.4)	94	(42.2)	161	(48.3)
NIPMS								
≤1 million	77	(25.9)	16	(7.3)	1	(0.4)	3	(0.9)
1-5 million	123	(41.4)	52	(23.7)	25	(11.2)	22	(6.6)
5-10 million	51	(17.2)	56	(25.6)	37	(16.6)	16	(4.8)
>10 million	45	(15.2)	95	(43.4)	160	(71.7)	292	(87.7)
unknown	1	(0.3)	0	(0.0)	0	(0.0)	0	(0.0)
OP first cycle	28	(9.4)	22	(10.0)	19	(8.5)	36	(10.8)
OP first finished episode	87	(29.3)	69	(31.5)	57	(25.6)	96	(28.8)
Nr of cycles in episode	3	(1-21)	3	(1-12)	3	(1-15)	3	(1-12)
Nr of cycles till pregnancy *	2	(1-11)	2	(1-9)	2	(1-7)	2	(1-9)

*based on the number of cycles with an ongoing pregnancy in the first finished IUI episode

OP= ongoing pregnancy. NIPMS= number of inseminated progressively motile spermatozoa. TPMSC and NIPMS are measured as volume × concentration × progressively motile spermatozoa

Table 3. The odds ratios of the sperm parameters and other IUI-associated variables for predicting the probability to become pregnant after the first IUI treatment of a couple and within their first episode, respectively, using univariable logistic regression

	n	First IUI cycle OR (95% CI)		First IUI episode OR (95% CI)	
Sperm morphology					
≤4%	444	1.19	(0.80-1.79)	1.39	(1.06-1.81)
>4%	629	1.00	(Ref)	1.00	(Ref)
TPMSC					
≤20 million	297	0.86	(0.51-1.45)	1.02	(0.73-1.44)
20-50 million	219	0.92	(0.53-1.61)	1.14	(0.78-1.65)
50-100 million	223	0.77	(0.43-1.38)	0.85	(0.58-1.24)
>100 million	333	1.00	(Ref)	1.00	(Ref)
NIPMS					
≤1 million	103	0.67	(0.30-1.51)	0.42	(0.23-0.76)
1-5 million	236	1.05	(0.64-1.71)	1.13	(0.81-1.57)
5-10 million	171	1.02	(0.58-1.79)	1.73	(1.21-2.46)
>10 million	655	1.00	(Ref)	1.00	(Ref)
Female age (per year)	1,166	0.93	(0.89-0.98)	0.91	(0.88-0.94)
Male age (per year)	1,166	0.96	(0.92-1.00)	0.95	(0.92-0.97)
Etiology female factor					
no female factor	737	1.00	(Ref)	1.00	(Ref)
female factor	386	1.35	(0.90-2.03)	1.24	(0.95-1.62)
Etiology unexplained					
no unexplained factor	750	1.00	(Ref)	1.00	(Ref)
unexplained factor	373	0.79	(0.51-1.23)	0.75	(0.56-0.99)
Nr of cycles in episode (per cycle)	1,166		NA	0.79	(0.74-0.85)

OR=odds ratio, CI=confidence interval, TPMSC=total progressively motile sperm count, NIPMS= number of inseminated progressively motile spermatozoa, Ref= reference, NA= not applicable

Table 4. The adjusted odds ratios of the variables associated with the probability to become pregnant after a first IUI cycle (n=1,166) and within the first finished IUI episode (n=1,072), using multivariable logistic regression

	First IUI cycle OR (95% CI)	First IUI episode OR (95% CI)	
Sperm morphology			
≤4%	-	1.52	(1.11-2.07)
>4%	-	1.00	(Ref)
NIPMS			
≤1 million	-	0.17	(0.09-0.32)
1-5 million	-	0.83	(0.57-1.22)
5-10 million	-	1.48	(1.00-2.21)
>10 million	-	1.00	(Ref)
Female age (per year)	0.94 (0.90-0.99)	0.92	(0.88-0.96)
Male age (per year)	-	0.97	(0.94-1.00)
Nr of cycles in episode (per cycle)	-	0.73	(0.68-0.78)

OR=odds ratio, CI=confidence interval, NIPMS= number of inseminated progressively motile spermatozoa, Ref= reference, - = not included in model
AUC first IUI cycle= 0.58 (95%CI (0.53-0.64)); AUC first IUI episode= 0.73 (95%CI (0.70-0.77))

It is interesting that the univariable model showed a higher probability of becoming pregnant in the first finished IUI episode for couples with ≤4% morphologically normal spermatozoa. In combination with the findings for NIPMS, this would mean that IUI is an especially relevant treatment for moderate male factor infertility, which is in accordance with most standards in infertility treatment (22). In contrast to our results, most studies have indicated that lower percentages of morphologically normal spermatozoa result in a lower probability of becoming pregnant (7-11,14,15,23). More important, the recommendations of some studies contradict our findings: couples with ≤4% normal sperm morphology have been advised to undergo IVF or ICSI instead of IUI (7,13).

Our results would intuitively make sense if infertile couples with good sperm quality were more frequently diagnosed with female factor infertility. The baseline characteristics of the study population underline this effect. Consequently, it is likely that the presence of female factors affects the relation between semen parameters and pregnancy outcome. When female factors are excluded, however, the probability of becoming pregnant based on semen parameters does not change in the opposite direction. Instead, the probability to become pregnant even increased in couples with ≤4% morphologically normal spermatozoa (OR 1.58; 95% CI, 1.13–2.22).

These apparently contradictory results raise the question of whether sperm morphology is an adequate predictor for the outcome of IUI. This view is supported by others (12,16,24). Note that it is also possible that the current WHO classification system may be inadequate (i.e., too strict criteria) but sperm morphology itself still could be an important parameter. It might be useful to consider the development of a new classification system.

Another striking result is that the TPMSC showed no prognostic value to predict the probability of becoming pregnant in couples with IUI treatment. Other studies have shown that TPMSC is of value when choosing between IUI and IVF or ICSI (17,25-27), and TPMSC has been described as a relatively good indicator for male factor infertility in general (27). Because of the lack of consensus, we additionally evaluated the threshold levels of the TPMSC as reported by others; we found no prognostic capacity (results not shown). In contrast to TPMSC, NIPMS was relevant in predicting the ongoing pregnancy rate after IUI. This is in agreement with the literature (2,4,6,10).

The final, multivariable model (Table 4) shows that the ongoing pregnancy rate in couples undergoing IUI was negatively influenced by a higher (>4%) percentage of morphologically normal spermatozoa, NIPMS of ≤1 million, higher female age, higher male age, and higher number of cycles within the episode. The predictive value of this model can be argued. Ideally, a prognosis is made between the fertility workup and the start of the treatment. At that time, the NIPMS and number of cycles within the episode are still unknown. When we excluded both variables from the final model, the receiver operating characteristic curve reported a lower discriminatory accuracy (AUC 0.73 toward 0.62). Furthermore, the predictive power hardly decreased when the sperm parameters were excluded from the final model (i.e., percentage of morphologically normal spermatozoa and NIPMS) (AUC 0.73 toward 0.69). Thus, this strengthens the conclusion that the predictive value of the sperm parameters for pregnancy outcomes in couples treated with IUI can be discussed. On the other hand, for most prediction models in reproductive medicine the discriminatory performance is low to moderate (28). From this perspective, every (small) addition toward a higher AUC is welcome.

To explain the different findings among the studies, some remarks can be made with respect to the study design and methods. First, several factors are related to the outcome of IUI (29-31), with the underlying etiology of infertility being one of them (32-34). Some researchers included couples with all etiologies in their study population. Among these latter studies, there was substantial diversity in the underlying etiology of infertility and the presence of different confounders and determinants within the study population (e.g., female age). Others stated that influences of semen parameters should be studied only in couples with male

factor infertility. All other etiologies of infertility should be excluded and the results corrected for confounding factors (27). These different policies with respect to the study population will likely result in different findings.

Second, among laboratories and clinics, there is wide variation in the methods of sperm preparation and semen analysis, the use or nonuse of different ovulation induction regimens, and the period of ejaculatory abstinence before IUI (4, 30, 35). In particular, the interpretation of the strict criteria for sperm morphology assessment has resulted in a decline over time in the reported percentages of morphologically normal spermatozoa (1, 36). In our study, we overcame this problem by selecting a study period within a methodologically stable period of time. Additionally, a longer time interval between fertility workup and the actual insemination might influence the adequacy of the prediction.

Finally, the way of applying statistics can lead to different results. Most studies did not take into account that the different cycles within a couple are not independent of each other and that, consequently, correlation could be present among the cycles within a couple. Standard statistical tests, however, lean on the assumption of independent observations; otherwise, they will result in other (too small) standard errors of the estimates, leading to incorrect significant results (21, 37). To overcome this problem of correlated cycles, we used the pregnancy outcomes in the first IUI cycle and in the summarized data of the first finished IUI episode. A downside of this method, however, is that it does not cover the variability of the individual IUI cycles. We recommend that future studies should analyze their data with adequate statistical methods, taking correlated data into account, such as the generalized estimating equations or multilevel analyses (38-40).

In our study, we took many factors of influence into account. Along with the methodologically stable period with respect to semen analysis and adequate statistics, we checked for the influences of ejaculatory abstinence, type of ovulation induction, and the time interval between the fertility workup and IUI on the probability of becoming pregnant. No effects were found (results not shown). Still, it would be valuable to set up a prospective, multicenter study with clear inclusion of couples, sound statistics, and standardized and controlled protocols for semen analysis, semen preparation, fertility workup, and the IUI procedure in general. This will provide an opportunity to develop a better prognostic model. Accurate study design also would be helpful for reconsidering the accuracy of the percentage of morphologically normal spermatozoa as factor for sperm quality assessment.

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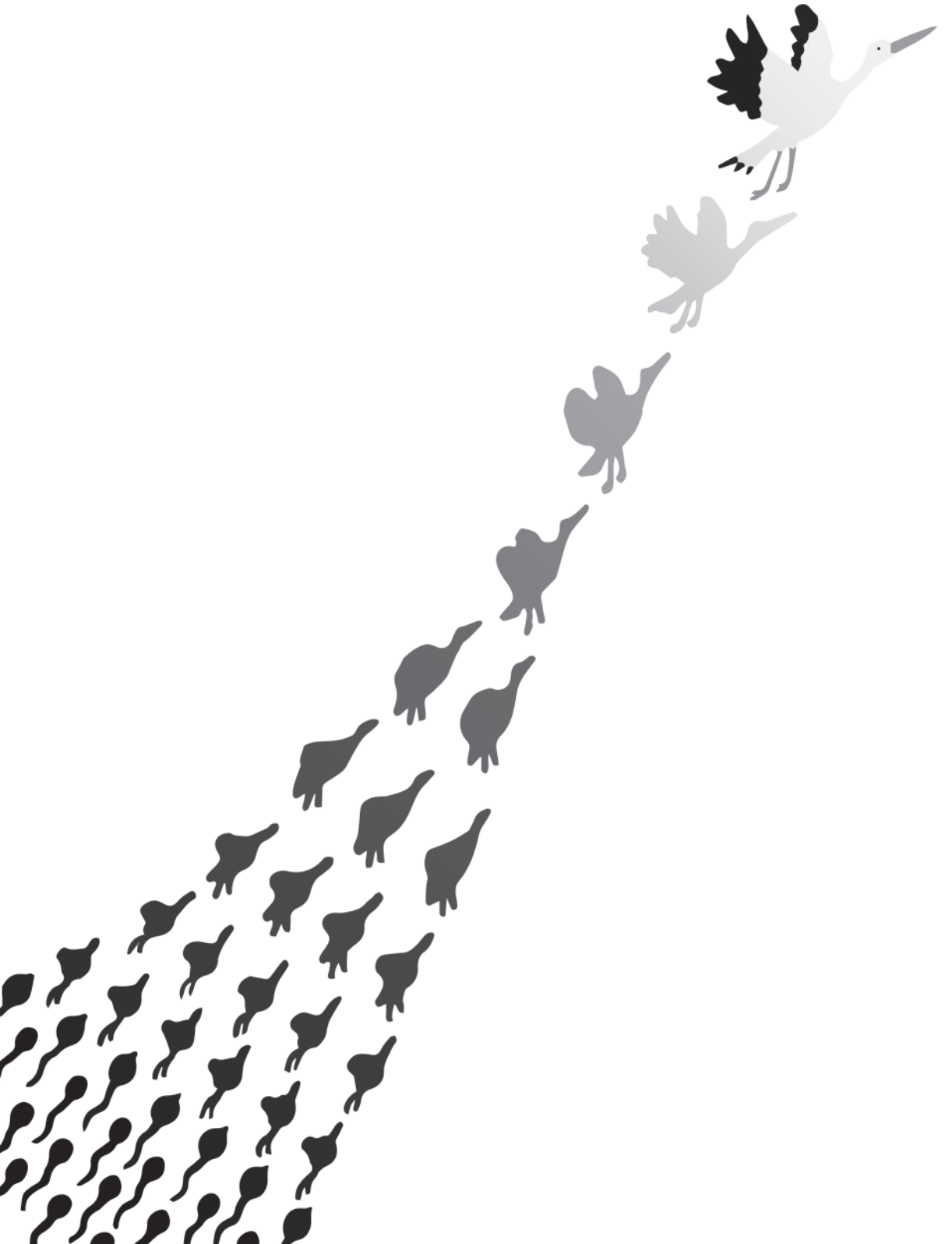

CHAPTER 5

External quality control and training of semen analysis in the Netherlands

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Submitted

Abstract

Semen analysis is characterized by high levels of intra- and inter- laboratory variability, due to a low level of standardization, high subjectivity of the assessments and problems with automated procedures. To improve consistency of laboratory results, quality control and training of technicians are important requisites. The goals of this study are to evaluate the results of an external quality control (EQC) program and standardized training by ESHRE Basic Semen Analysis Courses (BSAC) on the variability in manual assessments of semen parameters. We performed retrospective analysis of (1) inter-laboratory variability in the Dutch EQC program, and of (2) the inter-observer variability in BSAC's in The Netherlands, including the sperm parameters concentration, motility and morphology. EQC data showed that the inter-laboratory coefficient of variation (CV) for concentration assessment decreased (range of 24.0-97.5% into 12.7-20.9%), but not for morphology and motility assessments. Morphology assessment showed highest CVs (up to 375%), with many outliers in the period 2007-2014. During BSAC a significant reduction of inter-observer variability could be established for all parameters ($p<0.05$). Explanations for the absence of an effect on the CV in the EQC program might be found in the facts that motility assessment was introduced in 2008 and the criteria for morphology assessment changed. BSAC results might have been influenced by the pre-training level of participants and the influence of external factors. Both EQC and training show positive effects on reducing variability, despite the fact that laboratories have a low willingness to change their methods towards standards.

Introduction

The actual clinical value of semen analysis has been discussed for several years (1), due to, among others, a lack of standardization of the methodologies used for semen analysis and sperm preparation for fertility treatments (2,3,4,5) and the ongoing debate on the predictive value of sperm parameters for fertility (6). Even the introduction of laboratory manuals by the World Health Organization (WHO) since 1980 (7,8,9,10,11) did not result in the desired level of adherence and, over the years, the used methods remained highly variable between laboratories especially for morphology assessment (2,3,4,12,13). Next to standardization of used methods, another pitfall of semen analysis is the subjectivity of the assessment, characterized by intra- and inter-observer variability, as well as inter-laboratory variability. As a result, the quality of male infertility diagnostics might be impaired and, thereby, result in an inappropriate choice of treatment (14).

One strategy to measure intra- and inter-laboratory variability and the lack of standardization is to implement internal (IQC) and external quality control (EQC) programs. In different reports, EQC results showed a reduction of inter-laboratory variability over time for the three main aspects of semen analysis: sperm concentration, morphology and motility (15,16). IQC is an important requisite to improve consistency of analysis results within one laboratory, for example from one day of measurement to another (17,18). Both IQC and EQC are tools to evaluate whether procedures are effective, leading to a lower level of variability (19) and improved standardization over the years (16).

Training of technicians is another prerequisite to implement standardized methods and to minimize intra- and inter-observer variability. Reported immediate beneficial effects of semen analysis training were a substantial reduction of the variability in all aspects of semen analysis was reached within only a few days of training (14,22,23,24,25,26,27,28). On the long-term, training showed increased awareness of the need for standardization and even significant changes in used methods (27,29,30).

In the Netherlands, both quality control and training have been offered for several years. However, we showed in a previous study, that there is still a wide variability in used methods for semen analysis (4). Moreover, some of the recommended methods by the WHO were followed by rather small groups of the laboratories, for example the used counting chamber (~50%) and the staining method for morphology assessment (~10%) (4). Since the willingness of Dutch laboratories towards WHO recommendations was rather low, we expect that this could influence the impact of both requisites on the variability of semen analysis results. The objective of this study is therefore, to evaluate the impact over time on the variability of semen analysis results, shown by the Dutch EQC program and by the standardized training by the Dutch version of the basic semen analysis courses (BSAC) provided by the European Society of Human Reproduction and Embryology (ESHRE).

Materials and methods

EQC program

In the Netherlands, the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) distributes validated samples and digital data for the analysis of sperm characteristics, including the sperm parameters concentration, percentage of morphologically normal spermatozoa and percentage of motile spermatozoa (29). Participants of the regular EQC program are mainly fertility laboratories, clinical chemistry laboratories and microbiological laboratories. Since the start of the program, about 100 laboratories participate in the EQC program each year. Two samples for assessment of concentration, morphology and motility are distributed on a quarterly basis (i.e. March, June, September and December) and should, preferably, be evaluated by the technician in charge. In the period 2001-2018, 72 SKML distributions (144 samples) were assessed for sperm concentration and morphology as part of the EQC program. Motility assessment was included in the program since 2013; resulting in 48 samples assessed in 24 distributes in the period 2013-2018.

With permission of the local ethical board, EQC semen samples consisted of pooled semen that remained after semen analysis or IVF from men who visited the fertility laboratory of the Radboud University Medical Center and the clinical chemistry laboratory of the Sint Antonius Hospital. Semen used for concentration measures was prepared by density gradient centrifugation, diluted with Hayem's dilution (Boom, the Netherlands), stored at 4°C and sent to the participants in 0.4 ml aliquots. For morphology assessment, a small drop of semen (5 µl) was spread and air-dried on a microscopic slide. The participants were instructed about the preferred staining method. For motility assessment, semen was filmed in a Makler chamber at a magnification of x200 with phase contrast illumination. Each video consisted of two microscopic fields, each filmed for 40 seconds. Participants should evaluate motility of 200 spermatozoa of the samples according to WHO 1999 (i.e. rapidly progressive, slowly progressive, non-progressive and immotile) (8) or WHO 2010 (i.e. progressive, non-progressive and immotile) (7). Results of all participating laboratories were included in this study, irrespective of their used methods.

BSAC

To evaluate short-term effects of training, results obtained during BSAC were used. The BSAC has been given twice a year since 2008 at the Radboud University Medical Center. It has been offered to laboratory staff aiming to reduce variability and increase standardization of semen analysis. Course content is provided by the ESHRE Special Interest Group for Andrology (SIGA), is based on the prevailing WHO laboratory guideline and has been updated regularly in response to new

findings and publications (30). The training includes all aspects of standard semen analysis and lasts four days. A more detailed description of the training schedule and course content is described by others (25).

At the start of the course, participants assessed semen parameters (i.e. concentration, morphology, vitality and motility) of two samples, without having had any training (pre-test measurements). These parameters were assessed again for two samples after one day of theoretical and practical training (training measurements). At the end of the week, BSAC was completed with a practical examination, including assessment of all semen parameters of two more samples (exam measurements). It is important to note that different semen samples were used for the pre-test, training and examination and that the vitality results were not included in this evaluation study. All samples consisted of waste semen from men who visited the fertility laboratory of the Radboud University Medical Center.

Statistical analyses

Results of the EQC program, in the period 2001-2018 were presented as means and coefficient of variation (CV) per sample for all semen parameters. The results of all participating laboratories were used, irrespective of used methods. During BSAC, participants analyzed two different samples during pre-test, training and exam. The mean of both measurements was calculated. Accordingly, the CVs of all measurements per course were summarized in boxplots separately for the pre-test, training and examination measurements. The Friedman test was used for testing whether the results of the three measurements were statistically different. All statistical analyses were performed using SPSS IBM Statistics 22.0 for Windows.

Results

EQC program

Evolution of the inter-laboratory CVs of sperm concentration, morphology and motility assessment is shown in Figure 1. Additionally, the mean values of these measurements over time are shown in Figure 2. There was a decreasing trend for inter-laboratory CVs of sperm concentration assessment (range=24.0%-97.5% in 2001-2004), towards a more stable period in 2015-2018 (range=12,7%-20,9%). The variability in mean values had a more or less stable course over the total period (range=2.1-59.0). Morphology assessment was characterized by a declining trend in the means until 2006, whereafter multiple outliers (with a maximum of 375.0%) of inter-laboratory CVs were shown in the period 2007-2014. Since 2015, the range of inter-laboratory CVs has been stabilized (43.9%-69.6%). Both levels of inter-laboratory CV and mean (range= 15.0-95.0) of motility assessment variated constantly over the period 2013-2018.

BSAC

Results of sperm concentration, morphology and motility assessment during pre-test, training and examination are summarized in boxplots in Figure 3. In total, 19 courses were offered between 2008-2018, where 15 participants per course evaluated the semen samples. Between pre-test and training measurements, the CVs decreased significantly for all parameters (concentration: p=0.00; morphology: p= 0.00; motility: p=0.01). For concentration assessment, the initial improvement between pre-test (median=41.80) and training measurements (median=23.25) was followed by a significant deterioration (p=0.01) between training and examination (median=34.95). For both morphology (p<0.01) and motility assessment (p=0.04), the CVs significantly decreased from pre-test to examination. Differences between training and examination were not statistically different (p=0.64 and p=0.82, respectively).

Discussion

This study shows that, despite a low level of standardization, the variability in semen analysis results decreased in time in the Dutch EQC program, especially for concentration measurement. Also, during the Dutch version of the standardized BSAC of the ESHRE a positive effect of training on variability was perceived.

The need of standardization of semen analysis among laboratories was emphasized previously (2,31). Also, previous studies showed beneficial effects of EQC and training on realizing standardization of used methods and reducing variability of semen analysis (19,25,26,27). Despite the long-standing presence of the Dutch EQC program and BSACs in the Netherlands the used methods on fertility laboratories are still characterized by lack of standardization.4 This study showed that even with a lack of standardization, EQC outcome show a reduced variability of semen analysis results over time. Interestingly, when we only analyze concentration results of laboratories using the Neubauer chamber, reported CVs were comparable to the total CVs independent of used counting chamber (results not shown). The variability in semen analysis results might, therefore, not only be caused by the lack of standardization (i.e. in this case of the counting chamber). Training of the standardized method could, therefore, be an important tool to further improve BSAC results.

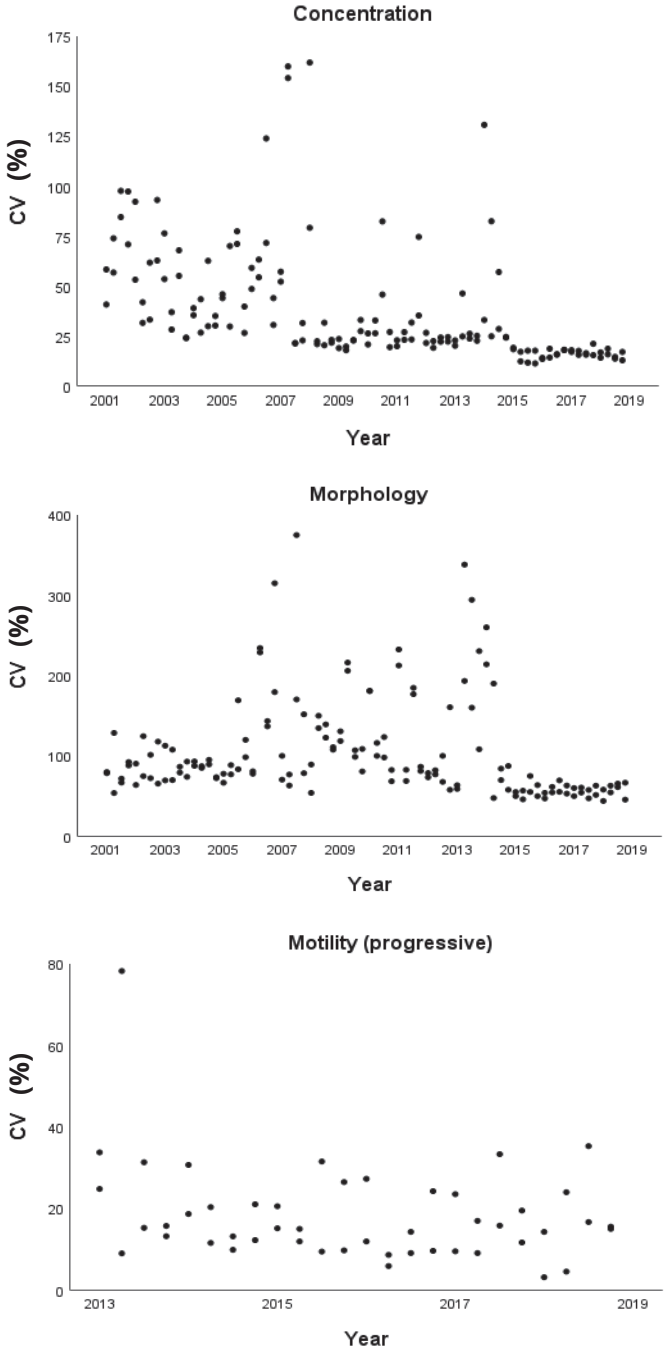

Figure 1. Overview of the coefficient of variation (CV) of the external quality control (EQC) program results for each semen parameter. EQC consisted of four distributions each year with two samples per distribution. Each dot represents the CV (in %) of one EQC sample between 2001 and 2018 (concentration and morphology) respectively between 2013 and 2018 (motility).

Where other studies showed reduction of variability of all aspects of semen analysis during EQC programs (15,16). our study only showed a positive effect on sperm concentration assessment. An explanation for the absence of a comparable effect for morphology assessment can be found in the instruction of using stricter criteria in the successive WHO manuals (7,8,9,10,11). The introduction of this criteria might result in some uncertainty among fertility laboratories, leading to an increased variability of morphology assessment results (multiple outliers within the period 2007-2014). This assumption is supported by the reported decline of assessed percentages of morphologically normal spermatozoa caused by methodological adaptations based on guidelines (32). As a result of this trend, the value of the assessment of morphologically normal spermatozoa in counseling individual couples is under discussion in recent literature (32,33,34).

For sperm motility, inter-laboratory CVs were comparable during the total study period. An explanation for this is that the motility assessment was included for a shorter period (since 2013) in the EQC program than concentration and morphology assessment (both since 2001). It is possible that the effect of EQC on the variability of motility assessment did already occur before 2013 and that this reduction is, therefore, not visible in the EQC results used in this study. Moreover, the CVs of motility assessment (<40%) were already lower than those of morphology assessment. Also, motility assessment in the EQC program was based on videos instead of microscopic examination, thereby limiting the influence of used methods (e.g. counting chamber and pipetting) on variability of the measurements. Compared to video assessment, it is known that real time microscopic motility determination of cryopreserved semen in EQC programs showed high inter-observer and inter-laboratory variability (35). Therefore, as long as WHO recommends real time manual motility analyses (7), the actual value of EQC video results will be lower than intended (lower commutability).

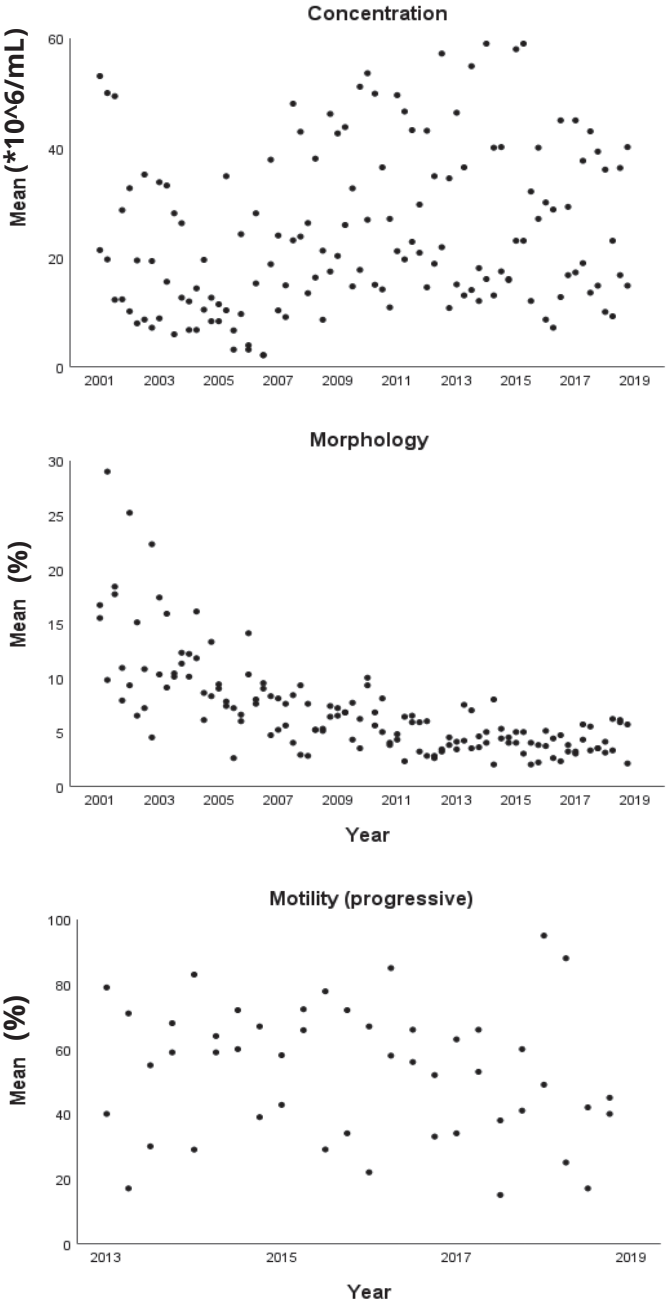

Figure 2. Overview of the measured mean values of external quality control program samples for each semen parameter. Each dot represents the mean value of the data supplied by all laboratories (one value per lab).

Next to EQC, another important requisite is training. BSACs have been offered since 1994, where the contents have been updated regularly based on new findings and publications (30). During standardized training courses, substantial reductions of inter- and intra- observer variability were reported, due to improved theoretical and technical skills of the participants (20,21,22,23,24,26). Also in the present study, a statistically significant reduction of the variability between pre-test (baseline measurement) and training was seen for all sperm parameters. For concentration assessment, however, this reduction was followed by a significant increased variability of examination results, where the variability between pre-test and examination results were not statistically different. This finding might be explained by the course contents and the manner the practical examinations of the course are set up. During pre-test, participants were asked to perform semen analysis using their own equipment and methods. The next day, WHO/ESHRE recommended methods were taught and trained in order to maximize standardization. For the final assessment, one hypothesis is that examination stress might influence the results, as well as increased time pressure (all assessments need to be performed within a time interval of two hours). Since concentration assessment is a complex analysis (36,37), we hypothesize that the impact of stress due to time pressure might be especially of influence on concentration results. This trend in concentration assessment, however, was not reported in a previous study (21).

Both EQC programs and training can be useful strategies to reduce variability of semen analysis and to implement standardization of used methods. It was shown, however, that even after introduction of both strategies, used methods for semen analysis are still characterized by a wide variability (4). In order to implement better standardization, it might be useful to improve both the offered training and EQC program. It is for example, important that the reported beneficial short-term effects on the variability of concentration, morphology and motility assessment caused by semen analysis training is also realized as long-term effects. It has already been shown that long-term effects of BSAC were an increased awareness of standardization and a change in used procedures towards recommended methods (25,27,28). As far as we know, however, the long-term influence of on-site training on the variability in EQC of sperm parameter assessment has not been evaluated before. Short (on-site) refresher courses based on BSAC courses and WHO guidelines following regular training programs might be an important requisite to realize long-term beneficial effects on variability of semen analysis. In this way, a more continuous training program is offered to fertility laboratories. The need for such approach is supported by the fact that we received frequent requests for (additional) on-site training from the participants of the EQC program.

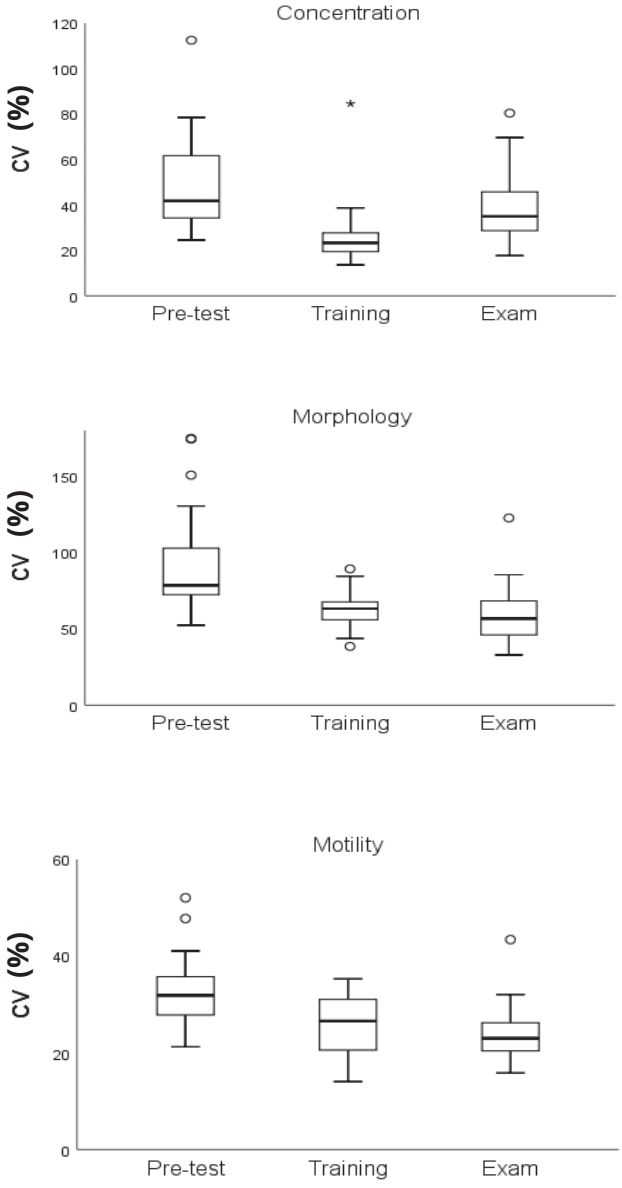

Figure 3. Boxplots of the coefficient of variation (CV) of pre-test, training and examination measurements for each parameter, during 4-day ESHRE basic semen analysis courses (19 courses with 15 participants each). Pre-test: results on day 1 of the course before any training; training: results during the training session; exam: results on the last day of the course during the examination.

Next to refresher courses, also the EQC setup can possibly increase awareness of variability in relation to standardization. In The Netherlands, the SKML introduced a multi sample evaluation system that is based on six sigma. This system indicates the performance of the participants by the precision and accuracy of their measurements. SKML assigns a performance score (0-2 points) for each semen parameter to indicate the agreement of the results compared to the reference values (mean values of the total group of participants) (38,39). The provided score informs the laboratories whether their results are within the target area. Scores of 1 and 2 indicate results of respectively 2 - 4.5 and ≥ 4.5 sigma within the "state of art" tolerance intervals. These scores are assigned per distribution and per year (multi-sample evaluation) (39). Reporting the EQC results in this manner might be more appealing to reduce the variability of semen analysis of the participating laboratories.

Overall, we conclude that reduction of both inter-observer and inter-laboratory variability is still an important challenge. Although many attempts were made, both by describing recommendations like WHO guidelines, semen analysis is still dependent on the willingness of fertility laboratories towards implementation of recommended or new methods. Approaches to reduce the lack of standardization of used methods did not yet result in the desired effect. Therefore, training, IQC and EQC are at the moment the most useful tools to reduce intra- and inter-laboratory variability of semen analysis. BSAC should be provided as an initial training program (21), followed by internal and external quality control programs, regular refresher courses and a course management system to maintain knowledge and to inform about new findings and publications. A combination of video instructions and an e-learning program could be an useful tool in this.

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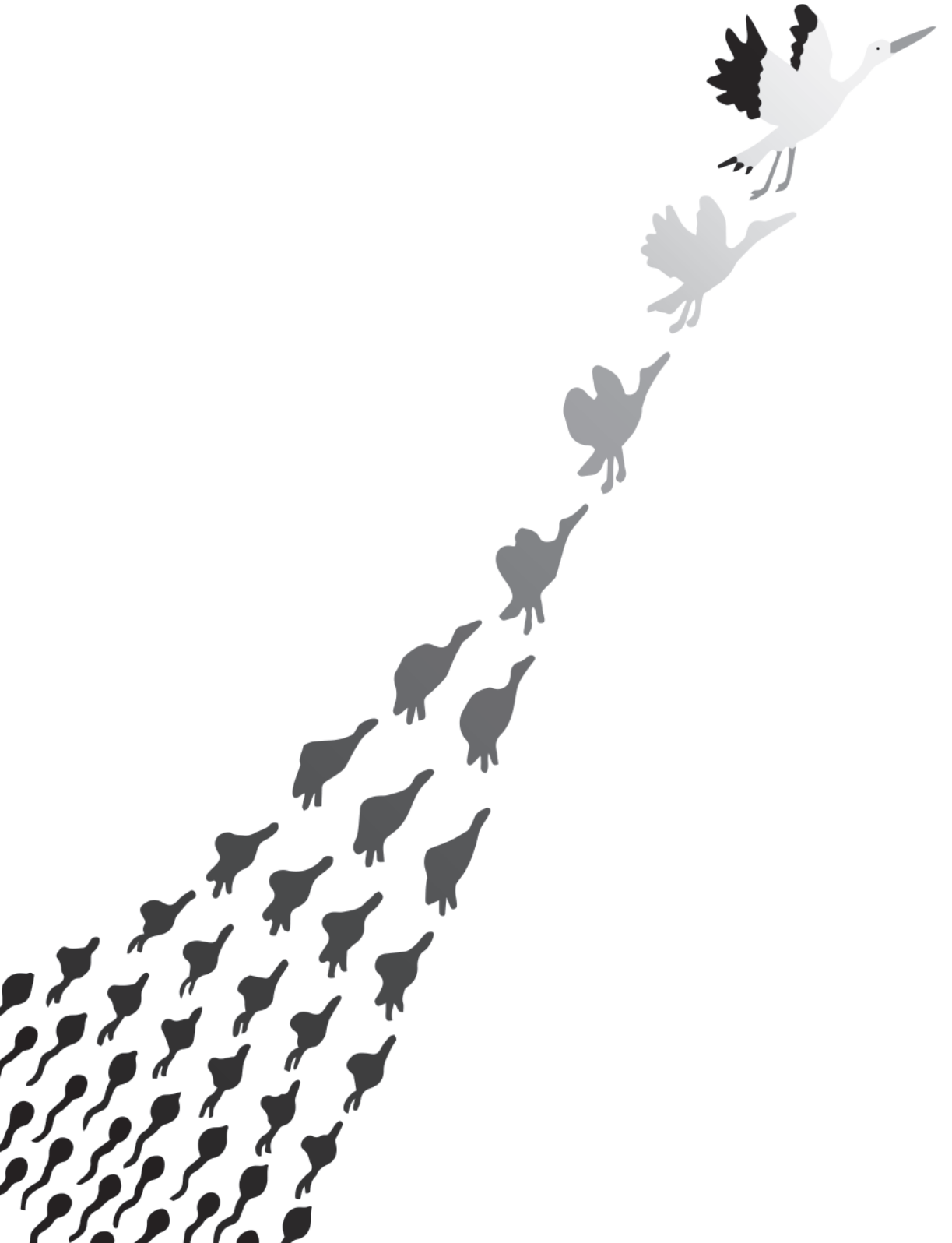

CHAPTER 6

Results of a short on-site training on semen analysis in the Netherlands

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Additional study to chapter 5

Introduction

The impact of external quality control and standardized training in the Netherlands on semen analysis results was described in an earlier study (1). Both are requisites to implement standardized methods and to minimize intra- and inter-observer variability. The EHSRE Basic Semen Analysis Course (BSAC) performed in chapter 5, has been offered since 1994 and the contents have been updated regularly based on new findings and publications (2). The standardized course was implemented all over the world, in the Netherlands since 1996 (3). This standardized training course improved theoretical and technical skills of the participants and, consequently, reduced inter- and intra-observer variability (2-6)). Chapter 5 did also show statistically significant reduction of the variability for all sperm parameters.

Where immediate effects of training were subject of several studies, long-term effects of training on variability of semen analysis results have not been studied before. However, training did show increased awareness of the need for standardization and even significant changes in used methods on the long term (3,5,7). Standardization of methods used for semen analysis is essential and it is, therefore, valuable to evaluate the impact on semen analysis results as well.

The working group on semen analysis of the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) received multiple request of Dutch fertility laboratories participating in the external quality control for medical laboratories for extra semen analysis training. They mainly requested for an update of insights in the field, recommendations on sperm parameter assessment and on-site training including subjects such as semen analysis and IUI. The goal of this chapter was, therefore, to provide short on-site training based on the WHO laboratory (8) and ESHRE recommendations and to evaluate its long-term effects on semen analysis results in an external quality control (EQC) program.

Materials and methods

EQC program

In the Netherlands, the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) distributes samples and digital data for the analysis of sperm characteristics, including the sperm parameters concentration, percentage of morphologically normal spermatozoa and percentage of motile spermatozoa. Participants of the regular EQC program are mainly fertility laboratories, clinical chemistry laboratories and microbiological laboratories. Since the start of the program, about 100 laboratories participate in the EQC program each year. Two samples for assessment of concentration, morphology and motility are distributed

on a quarterly basis (i.e. March, June, September and December) and should, preferably, be evaluated by the technician in charge. Motility assessment was included in the program since 2013.

Since 2015, the results of the SKML participants are reported in the six sigma context, indicating their performance by the precision and accuracy of their measurements. SKML assigns a performance score (0-2 points) for each semen parameter to indicate the agreement of the results compared to the reference values (mean values of the total group of participants) (9,10). A score of 0 points means that the results are below 2 sigma within the target area. One and two point scores indicate results of respectively 2 - 4.5 and ≥ 4.5 sigma within the "state of art" tolerance intervals. These scores are assigned per distribution and per year (multi-sample evaluation) (10).

On-site training

In 2015, a total of 106 laboratories participated in the regular EQC program of the SKML. All laboratories were invited for on-site semen analysis training. Interested laboratories performing all analyses of interest (i.e. concentration, morphology and motility assessment) were randomly divided into a training group (n=8) and a control group (n=16) without training. The training was given in Dutch language and was developed for experienced technicians performing semen analysis. Training took approximately 2-4 hours and included fundamental principles of semen analysis, as well as information about the EQC program of SKML. The content of the training was based on the laboratory manual of the WHO (8) and the recommendations provided by the ESHRE SIGA (11). Also, used methods and materials of the laboratories were evaluated and, if necessary, suggestions to improve these methods were provided. After all training visits took place, a list with recommendations based on observations or questions raised during the visits was sent to the participants of the training group.

In order to realize standardization, the laboratories in the training group were asked to analyze the post-training measurements by standardized materials and methods. Participants were recommended to use a positive displacement pipette for sampling and the improved Neubauer haemocytometer for sperm counting. For assessment of the percentage morphologically normal spermatozoa, use of the May-Grünwald-Giemsa staining (MG Giemsa) (12) was recommended, since this was the best available staining method on the visited laboratories.

To evaluate the impact of on-site training on the accuracy of semen analysis, results of the EQC program were used. Since all laboratories in the group were visited and trained in January-February 2016, results of the four measurements

in 2015 were used as baseline measurements. They were compared to the four (post-training) measurements in 2016.

Statistical analyses

Boxplots and SKML scores (0-2) were used to examine the impact of on-site training on semen analysis results. Measurements of each sample were separately shown for the groups with and without training. A mixed-effects analysis was performed to test if participants of the training group performed sperm assessment with less variability after training compared to before the training. Training (yes/no) and moment of measurement (before/after training) were evaluated as fixed-effect covariates and lab number, distribute (1-8) nested in lab and sample (A/B) nested in distribute were included as random effects. All statistical analyses were performed using SPSS IBM Statistics 22.0 for Windows.

Results

In total, 8 laboratories were included in the training group and 16 laboratories in the group without training. The methods used before and after training for both groups were summarized (Table 1). Strikingly, although clear instructions were given, training did not result in important shifts in applied methods. The SKML performance scores (score 0,1 or 2) did not change significantly: the intervention group scored on average 72% (SD: 14) of the maximum achievable score in 2015 and 78% (SD: 11) in 2016, where the control group scored 68 (SD:21) and 80 (SD: 17), respectively. Also, boxplots (Fig. 1) show that the agreement between laboratories in both groups (training and control) was comparable for sperm assessment before (samples 3/'15 – 12/'15) and after (samples 3/'16-12/'16) intervention. Moreover, there was no difference in sperm assessment between the groups with and without training. Results of the mixed-effects model were in accordance with this: sperm parameter results were not influenced significantly by participating in the on-site training program (concentration: p= 0.98; morphology: p= 0.22; motility: p= 0.21).

Table 1. Semen analysis procedures used by the participating laboratories in the intervention (training) and control (no training) group during the SKML distributions in 2015 (before intervention) and 2016 (after intervention). The numbers represent the number of laboratories using a certain technique.

	Before intervention	After intervention
Training group (n=8)		
Counting chamber (concentration)		
Improved Neubauer	6	6
Makler	1	1
Bürker Türk	1	1
Staining method (morphology)		
Diffquik	6	5
MG Giemsa	1	2
Papanicolaou	1	1
Classification WHO (motility) *		
WHO 2010	6	7
WHO 1999	3	2
Control group (n=16)		
Counting chamber (concentration)		
Improved Neubauer	9	10
Makler	2	2
Bürker Türk	3	2
CellVision	1	1
Other	1	1
Staining method (morphology)		
Diffquik	13	13
MG Giemsa	1	0
Papanicolaou	1	1
Other	1	2
Classification WHO (motility) *		
WHO 2010	5	7
WHO 1999	12	11

*Some laboratories evaluated the motility according to both WHO 1999 and WHO 2010 and/or changed their methods over the different measurements.

Discussion

The short-term beneficial effects of standardized training reported in chapter 5, seem to be temporary as long-term effects of on-site training on the results of EQC could not be measured (Fig. 1). To draw a hard conclusion, however, it is important to take the differences in the setup of both training programs into mind. BSAC lasts 4 days, the program is interspersed with several breaks, it is offered to technicians from different laboratories and their acquired skills were tested during examination. In contrast, on-site training was short (up to 4 hours), offered to technicians of one laboratory using their own equipment and participants were not tested during an exam.

Sensemaking is an important process to achieve changes in professional environments (12) and could be the most important factor causing the reported differences between short-term and long-term training results. Sensemaking is about the ways people generate what they interpret and how gathered information takes form when people make retrospective sense of this, based on their own experiences and consequences of their actions (13). It is an ongoing social activity in which individuals respond to the environment they face (14). This process could explain the differences in effectiveness of the two trainings offered in our study. Where sensemaking is present in BSAC since the training lasts 4 days and includes time for social interaction, the short and top-down instruction provided during on-site training is missing this aspect. Lack of sensemaking could be responsible for unchanged variability after training, but also for the fact that most laboratories with training did not change their used methods (Table 1). Although on-site training could be useful, the setup should be evaluated and adjusted, taking sensemaking into account. Related to this sensemaking theory is the necessity of internal quality control (IQC) and in-house training. Once qualified for analytical work according to the standards of a center, a technician should participate in ongoing IQC and, if necessary, get in-house training.

Other reasons for the lack of effect of on-site training on long-term results, can be found in the selection procedure of participating laboratories, the statistical power of the study and geographical limitation. Selection was random, as well as the randomization into training and control group. This resulted in more or less comparable pre-training results between both groups. Since knowledge and experience of the technicians impacts the reliability of semen analysis results (15), on-site training might have resulted in a larger effect when offered to laboratories with lower baseline results. The statistical power of the study is too low to draw proper conclusions based on our findings, also as a consequence of the high variations in results. This study can, however, be seen as a pilot for future, more extensive research. Geographically, the study is limited to The Netherlands. Earlier research showed that many Dutch laboratories are not prepared to work strictly according to WHO instructions (16). Because this is not a unique situation (17-19), our results can be valuable worldwide.

Training of fertility technicians is an important tool for reducing variability of semen analysis. However, the short on-site training offered in this study was not sufficient for realizing long-term beneficial effects. It seems useful to put more time and effort in developing a more extensive training program. BSAC could be used as initial training (2), followed by quality control programs and multiple regular refresher courses. Update of findings and new publications should be taken into account. Such approach needs to be subject for further research.

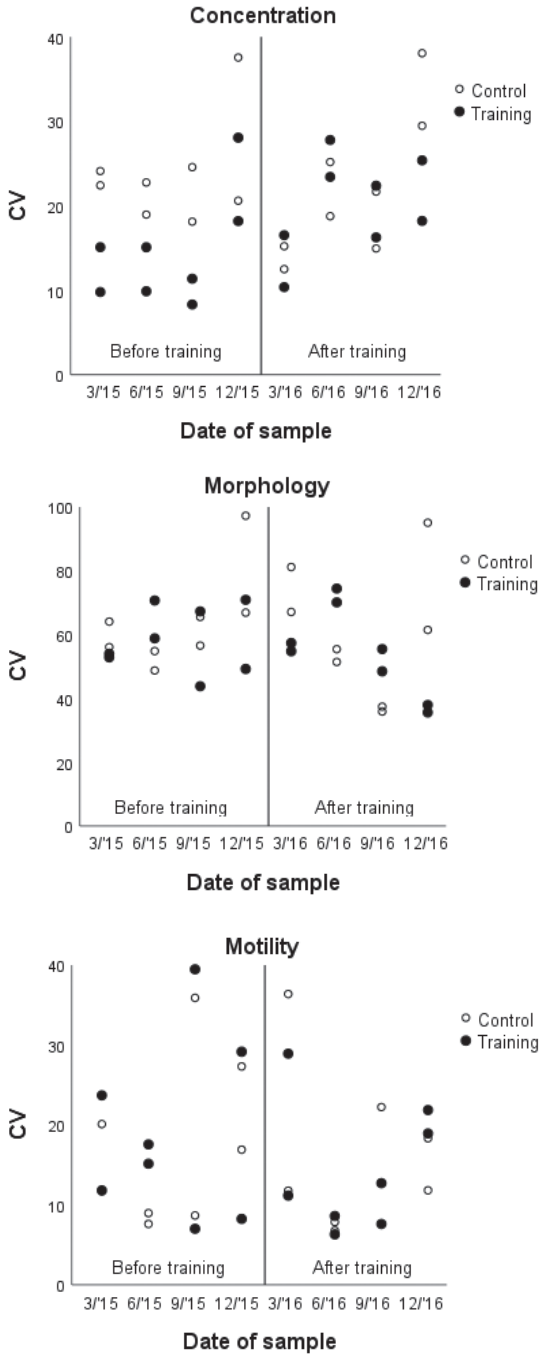

Figure 1. Coefficient of variance for each parameter of two different samples assessed before training (March, June, September, December 2015) and after training (March, June, September, December 2016), for the training group and the control group without training.

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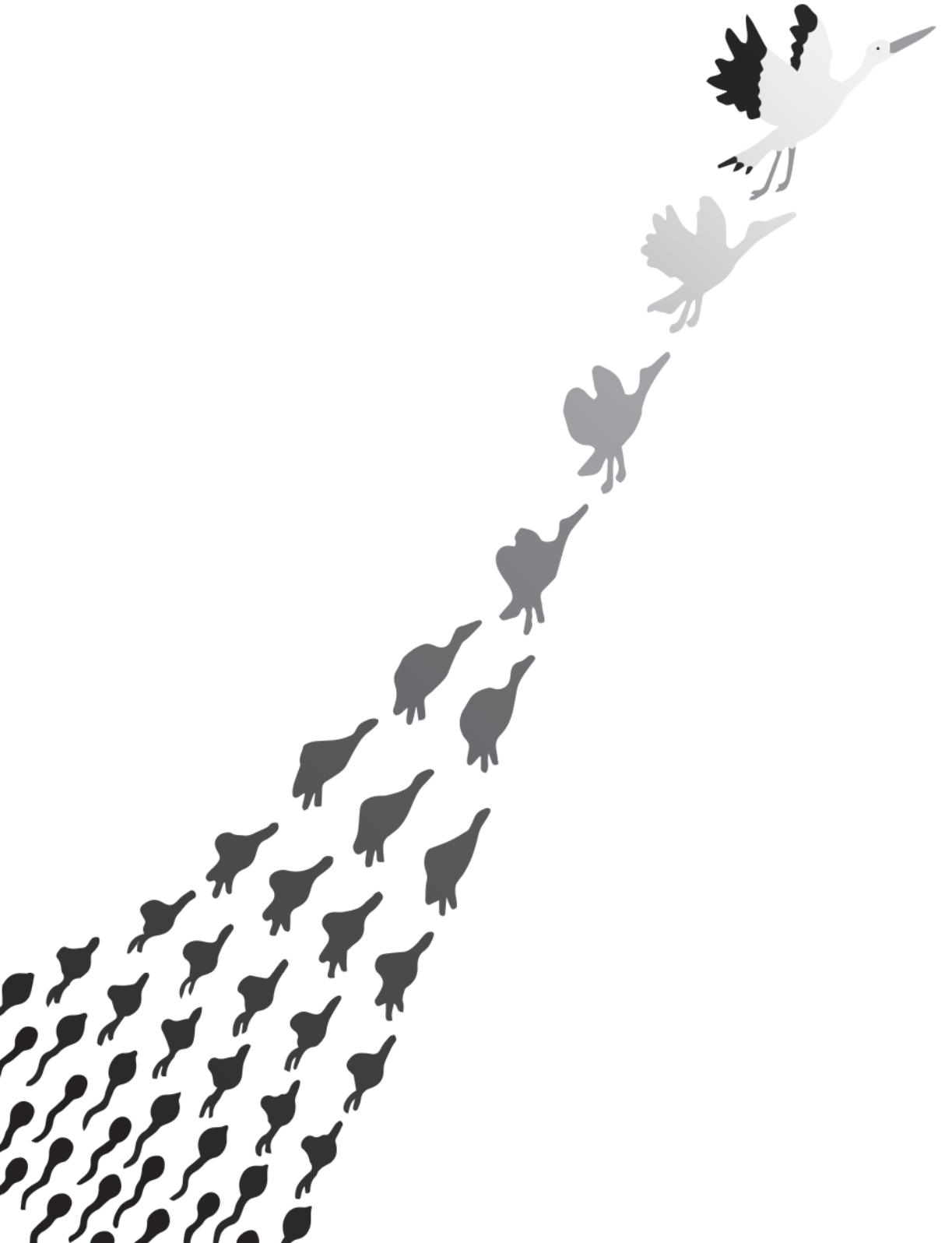

CHAPTER 7

General discussion

Infertility has a high impact on the quality of life of an affected couple, resulting (not exclusively) in social, sexual, psychological and emotional consequences. Also, higher rates of depression, nonspecific emotional distress, less life satisfaction and happiness, anxiety and even episodic suicidal ideation are reported in couples seeking fertility treatment (1). It is, therefore, important to provide an adequate infertility diagnosis, to minimize the impact of infertility treatment and to optimize the pregnancy results of the offered treatment. One way to realize this is optimization of semen analysis and (the technical phase of) IUI.

Semen analysis is an important tool to investigate male fertility and is, therefore, an important requisite for selecting the best treatment option offered to an infertile couple. IUI is one of the treatment options for infertile couples and is cost-effective in selected couples. However, due to lack of evidence-based recommendations and lack of standardization for both aspects, improving both techniques is an important goal to pursue. Basically, this thesis aimed to provide recommendations for fertility clinics to improve semen analysis and IUI for the treatment of couples diagnosed with male factor infertility.

Evolution of IUI

Louise Brown, born in 1978, is known for being the first human conceived after using IVF. The story about the first child born after the use of donor insemination, however, is less well-known. Professor Pancoast performed, back in 1884, the first artificial impregnation of a woman. Without knowledge of the examined infertile couple, the professor inseminated fresh semen from the "best-looking" medical student, which resulted in the birth of a boy (2). Presumably, it was the start of the use of donor insemination. Consequently, the development of artificial insemination methods was described for the first time in 1899 (3). Several new techniques have been developed and introduced since then, including the sperm preparation, use of frozen spermatozoa, follicle stimulation and the timing of ovulation. The popularity of artificial insemination, however, increased most after the introduction and availability of donor sperm (3). Due to improved sperm selection techniques, IUI became the first choice of treatment in selected infertile couples.

The success of IUI is dependent on several factors, such as female age (4,5), duration of infertility (6), stimulation protocol (7), timing of IUI (8) and more. The impact of patient characteristics and clinical factors on IUI outcome has been studied extensively (9,10). On the other hand, the technical stage of IUI has been studied less frequently and is barely included in clinical guidelines. So, where the optimization of artificial insemination methods took a run over the years, efforts to optimize basic procedures used during IUI treatment more or less stagnated. Only the WHO published a guideline including recommendations, focusing on

the subjects semen analysis, sperm preparation and quality assurance (11). The previous edition of the WHO guideline (1999) was updated in 2010, with major changes on sections about cryopreservation of spermatozoa, assessment of semen analysis parameters (i.e. sperm numbers, azoospermia, motility and morphology) and quality control (11). Updating the current knowledge among procedures performed during the technical stage of IUI could result in standardization of used methods, but also in improving the effectiveness of IUI treatment.

Chapter 2 of this thesis summarizes all available literature focusing on the influence of procedures related to semen collection (e.g. ejaculatory abstinence, collection place), semen processing (e.g. preparation method, buffer of wash medium) and insemination (e.g. method of timing, bed rest after IUI) on pregnancy results of IUI. Also, the association between pregnancy rates and several time intervals related to the IUI treatment and the used equipment were evaluated. This review revealed, unfortunately in accordance with the expectations, that it is still hard to come-up with evidence-based recommendations in order to develop guidelines for optimal IUI treatment. The problem related with these IUI procedures is threefold: 1) for some procedures only limited studies were performed and/or well-designed retrospective studies are not available 2) available literature is characterized by the absence of standardized methods or the inclusion of small study populations, 3) impact of the procedures on pregnancy results has not been studied yet, only the impact on for example sperm parameters or other pregnancy related factors.

It has already been shown that the willingness to implement guideline recommendations in IUI care is far from optimal (12,13), which mainly could be explained by missing supporting evidence for the provided recommendations (14). Unfortunately, we were also unable to endorse earlier provided findings, even after an extensive literature search on this subject (chapter 2). Whether the lack of standardization related to this could also be seen in the Netherlands was evaluated in chapter 3. An overview of the used methods for both semen analysis and laboratory IUI procedures at that moment was provided, based on a survey performed at Dutch fertility laboratories. Only a limited number of the participating laboratories used the procedures as recommended by the WHO guideline (11), which was especially the case for semen analysis methods. As a result, a wide variation of used methods was seen. Again, the need of standardization of IUI methods was emphasized, also supported by the reported influence of the investigated procedures on IUI pregnancy results. Even though their impact was analyzed without taking care of any bias or the predictive power of reported associations, it indicated that standardizing these methods will help optimizing performance of IUI care among IUI clinics.

As stated before, used IUI procedures lack standardization and used methods remained more or less the same over the last years, mainly as a result of a lack of evidence of recommendations in this field. This does, however, not mean that the question "techniques used for IUI: is it time for a change?" has never been raised before. Several research groups put effort in evaluating specific parts of IUI and the impact of used methods on its outcome. However, as long as performed studies fail to show clear evidence in favor of new or not yet standardized methods compared to the currently used methods, lack of standardization and unwillingness to adapt laboratory methods will continue to exist. Based on earlier publications of their guideline, the WHO was aware of the fact that some recommendations needed sufficient explanation and supporting evidence. In many cases, however, they experienced difficulties with endorsing, changing or updating them, due to insufficient available data (11). Along with that, our recommendations provided in chapter 2 were perhaps not always supported with sufficient evidence, but should be considered for other important reasons (i.e. for the purpose of standardization, ease, quality control or costs). Even more, the results of our questionnaire study (chapter 3) pointed in the same direction of optimal procedures.

Missing evidence-based recommendations is not the only reason that the willingness to adapt current procedures is rather low. The value of IUI in infertility treatment management is under discussion. For several years, IUI was reported as first-line treatment in couples suffering from mild to moderate male factor and unexplained infertility (15). IUI is a relatively simple and non-invasive treatment, reported as being cost-effective in these specific couples. In 2013, however, the NICE published a guideline where expectant management was recommended over IUI in couples with unexplained infertility, mild endometriosis or mild male factor infertility (16). They stated that these couples should be offered IVF after they have attempted to conceive for a total of two years by having regular unprotected sexual intercourse. IUI should only be offered as a treatment option to couples being unable to have vaginal sexual intercourse, for example due to clinically diagnosed physical disability or psychosexual problems, in couples with specific conditions (e.g. HIV positive male) or in same-sex relationships (16).

Even before the NICE guideline was updated, the effectiveness of IUI compared to IVF and ICSI success has been discussed extensively (17-20). Around development and publication of NICE guidelines, there were even a few studies reporting evidence against the use of IUI (21-23). The recommendations of the NICE, however, have led to a lot of commotion in the field, with the general conclusion that the recommendations on IUI procedures need a radical review (3,24-26). Several studies investigated the value of IUI since then and concluded that it is still a useful, cost-effective first-line treatment in selected couples, especially those with mild male factor and unexplained infertility (27-30). Moreover, a large

majority of fertility clinics (96%) in the United Kingdom continued to offer IUI after the publication of the NICE recommendations (25,31,32). As a response to the provoked debate, the NICE reviewed available evidence on their recommendations on IUI treatment. They concluded, however, that there was no reason to adapt earlier provided recommendations (33).

Based on our findings, the conclusions drawn by the NICE seem to be too short-sighted. IUI can still be an important requisite in the treatment of couples suffering from infertility. Even more, IUI pregnancy results could be improved by several factors (i.e. by knowledge expansion and standardization (chapter 2 and 3)). The low willingness to adapt current protocols is understandable, especially when evidence-based recommendations are missing. Discussion regarding the effectiveness of the treatment could also clarify why clinics are less willing to change their way of working and to put effort in related costs and time. Participants of our survey (chapter 3), however, informed us that they recently changed or were planning to change parts of performed semen analysis and/or IUI as a result of re-organization (e.g. by fusion of laboratories. This should be a perfect moment to thoroughly evaluate currently used methods and associated potential improvements of it. Nevertheless, they should take into account that the use of standardized methods is important for optimizing the performance of care, counseling of infertile couples and IUI related pregnancy results.

It is not only important to provide evidence-based recommendations in available guidelines, but also to apply adequate strategies to implement them in clinical practice. The difficulties associated with guideline implementation are related to personal factors, guideline-related factors and external factors (34,35). Where strategies are successful in one setting, they may be less successful in another setting with different barriers (35). Implementation strategies are more likely successful when the barriers are analyzed in advance and when the performed strategy includes different types of interventions and addresses physicians' knowledge and attitudes (34).

Realizing standardization of IUI

To realize standardization, the main goal of chapters 2 and 3 was to enlarge the level of knowledge available for semen analysis and IUI procedures. Another way of providing tools for realizing standardization is by an practical approach of knowledge gaps that are still present for individual semen analysis and IUI related factors. Two examples of these factors were investigated in chapters 4 (i.e. sperm parameter assessment), 5 and 6 (i.e. training and quality control). The main reason for analyzing those two subjects, was a result of the input of Dutch fertility laboratories participating in the program of the Dutch Foundation for Quality

Assessment in Medical Laboratories (SKML). The semen section of this nonprofit organization for external quality control for medical laboratories received during the last couple of years multiple requests for either updated insights and recommendations on sperm parameter assessment (most frequently concerning assessment of sperm morphology) and the possibilities of on-site training about semen analysis and IUI.

Sperm parameters have been reported to affect pregnancy rates of IUI. Higher pregnancy rates were reported for semen assessment with a higher level of total motile fraction (4,6,36), larger baseline spermatozoa concentration (37) and higher percentage of morphologically normal spermatozoa (36). Since the introduction of the strict criteria, however, the value of sperm morphology assessment has been subject of discussion. In the Netherlands, only a small majority of laboratories still performs sperm morphology assessment during routine semen analysis in 2016 (chapter 3). During counseling of couples, the validated Hunault model is used in Dutch fertility clinics (38,39). Sperm morphology is not included in this model, which makes it less relevant to perform assessment of this parameter or to perform it accurately. The predictive power of the percentage of morphologically normal spermatozoa on IUI pregnancy outcomes has been subject of discussion since the use of the strict criteria (33,40-49). About the predictive value of other sperm parameters has also been some disagreement, yet less frequently. The total progressively motile sperm count (TPMSC) was reported to have discriminative value (50), however, only with high specificity for identifying those who are unlikely to become pregnant after IUI (51-53). A minimum of 5 million inseminated progressively motile spermatozoa (NIPMS) is required for IUI, but the predictive value of this parameter is less clear (54,55).

The predictive value of percentage morphologically normal spermatozoa, TPMSC and NIPMS for IUI ongoing pregnancy was evaluated in couples visiting the fertility clinic of the Radboud University Medical Centre (chapter 4). In predicting the pregnancy chance after the first IUI cycle, none of the sperm parameters turned out to be of value (i.e. only female and male age). On the other hand, a percentage of morphologically normal spermatozoa <4% and a NIPMS between 5-10 million were found with a higher probability to become pregnant after first IUI episode. In combination with other known predictors of pregnancy, only sperm morphology remained of importance. Even though the predictive power of the included sperm parameters was rather low, we concluded that both performing semen analysis and offering IUI to couples suffering from moderate male factor infertility should still be considered as relevant (i.e. sperm morphology ≤4%, NIPMS 5-10 million).

The value of morphology as parameter to predict IUI pregnancy outcome was by others reported as less valuable compared to the predictive value of other sperm

parameters, but it does not mean that the evaluation of sperm morphology would be of none value. Problems related to morphology assessment are the subjectivity of the measurement, lack of compliance towards WHO standards and the low reference value of 4% morphologically normal spermatozoa. The introduction of this reference value was based on the definition for morphological normal spermatozoa based on spermatozoa obtained from the level of the internal cervix after penetration through cervical mucus (56). However, when classification was doubtful, the spermatozoa should be classified as abnormal (57). The use of strict criteria has been criticized for several reasons (58). One of them was that the majority of motile spermatozoa are incapable of binding to the zona pellucida, which indicates that male subfertility is related to a reduced number of capable sperm instead of reduced capability in all sperm (59). Also, a morphologically "normal" looking spermatozoon does not necessary have the ability to fertilize an oocyte, if capacitation or the acrosome reaction does not occur (60). However, abnormal morphology results should be an indication to evaluate the health of the male partner (e.g. by testicular ultrasound or endocrine examination). Moreover, specific morphologically abnormalities, such as globozoospermia or stress-induced elongated sperm heads, will be missed during sperm morphology assessment. While these abnormalities are rare, ignorance does have impact on adequacy of patient selection for fertility treatment (57).

Studies performed after publication of chapter 4 reported better pregnancy results in the group with normal sperm morphology and higher progressive motility of the spermatozoa (61,62), but also no impact of TPMSC on IUI pregnancy rate (152). Also, a review concluded that sperm morphology did not affect IUI pregnancy outcome (154). Concerning the NIPMS, results suggested importance as predictor of IUI pregnancy. Nevertheless, NIPMS is not assessed during initial fertility workup and would therefore only be useful determining whether a couple should move over to another fertility treatment option once IUI has already been started (55). An already known pitfall related to fertility studies is also applicable for studies evaluating predictive power of sperm parameter assessment: lack of standardization in study designs, in methods used to perform semen analysis, in included study patients and in policy regarding other predictive factors (50). This could not only be the explanation for reported conflicting results, but also in an overall over- or underestimation of the predictive value of (one of) these parameters. Overall, we showed the importance of assessing values of sperm parameters during semen analysis, since they provide useful information for counseling of male factor infertility. Combining the values of all parameters could also provide clinically meaningful classification into fertile, indeterminate and subfertile (65). However, also in this classification system overlap was reported, probably due to female factors contributing to the infertility of the couple (65).

Where chapter 4 was set up to contribute to knowledge enrichment regarding parameters assessed during semen analysis, it is important that this information should reach the designated persons. Training of laboratory personnel is one important requisite to reach this goal. Also, it is a useful strategy to implement standardized methods and to reduce variability of semen assessment results caused by the subjectivity of the assessment (66). Intra- and inter-observer variability and inter-laboratory variability have impact on the reliability of counseling male factor infertility and, consequently, on the adequacy of treatment selection (67). Reducing variability of all aspects of semen analysis could already been reached by training of laboratory technicians within only a few days (67-72). On the long-term, most important consequences of training were laboratories changing their methodologies and trying to reach standardization (73-75). These studies, however, were already performed over twenty years ago, long before the last update of the WHO laboratory manual.

The training program (basic semen analysis courses (BSAC) organized by the European Society of Human Reproduction and Embryology (ESHRE)) was standardized and implemented in several countries throughout the world (69). Since 1996, the course has also been offered in the Netherlands (75). During this training, a theoretical background and extensive practical training regarding semen analysis is given. Semen parameters were assessed at three moments during the training program (pre-test, during training and examination). We found in chapter 5 that a reduction of the variability of measurements was, in particular, seen between pre-test and training measurements. This trend was not continued in the same manner towards examination, probably caused by examination stress and the way examination was performed. On-site training, based on the BSAC courses, was offered to Dutch laboratories as additional training moment (chapter 6). The impact of this training moment was evaluated during four measurements over time between laboratories with and without training. There was no significant influence of training on the variability reported for any of the assessed semen parameters. Also, the training program did not result in standardization of used methods in the training group. The absence of long-term effects, however, could also be related to the design of performed training program. Reasons for the lack of long-term effects of on-site training might be found in lack of standardized procedures of participating laboratories, low statistical power to the small sample size, too concise contents of the course or lack of sensemaking introduced in the course. Sensemaking is important, since it influences the way of interpretation of the course contents (76) and it determines the way participants will respond to it (77). Laboratories will have more willingness to change their used methods, when the training includes adequate sensemaking.

The implementation of an external quality control (EQC) program is another approach to measure intra- and inter-laboratory variability and may, consequently, reduce practice variation. Next to reducing inter-laboratory variability over time (78,79), it caused also a reduction of inter-observer and intra-laboratory variability (80,81). Together with appropriate training of personnel, internal and EQC programs should, therefore, be considered as standard policy on fertility laboratories. This statement is supported by our findings for evaluating results over time (2001-2018) of the Dutch EQC program in chapter 5. Especially for inter-laboratory variability of sperm concentration, the reduction over time was important. On the other hand, such decline could not be found for morphology assessment. This was most probably caused by methodological changes performed in this period.

In general, semen analysis methods did hardly change over the years. Comparing methods recommended in the current WHO guideline (11) with those provided in the first version of the guideline (published in 1980) (82) demonstrates that the majority of the recommendations remained the same. Where automation was implemented in clinical chemistry and microbiology, this is not the case for semen analysis. There were only a few attempts to introduce automated procedures in this field. Using automated procedures, however, eliminates variability caused by manual assessment and it is therefore valuable to develop or improve such procedures (83,84). Future studies could for example focus on computer assisted semen analysis (CASA). It was introduced in the early 1990s and has still disadvantages with respect to manual analysis, in terms of precision, accuracy, know-how of the technician and analysis time (85). As a result, large groups of experts share the opinion that manual analysis should be preferred and strict standards must be set before the implementation of CASA (86). Others believe in the benefits of using CASA when further evaluation is performed for morphology assessment (87) or parameters other than routinely performed according to the WHO are measured, like principal piece analysis (85,88,89). Before CASA could be implemented in routine semen analysis, more research is needed. Another automated procedure valuable as subject for future studies is microfluidic spermatozoa selection. Microfluidic technologies could be used for the analysis and separation of sperm cells (90-92). Next to potential value for diagnostics, it was shown that these technologies could be used to separate boar spermatozoa from erythrocytes (93). This can be useful for ICSI procedures, in cases where sperm has to be extracted from testicular biopsies or epididymal aspirations. However, before this chip can be validated and, consequently, be used in the clinic, further experiments are needed to optimize this chip and to make the system suitable for clinical applications.

Recommendations for the fertility clinic

The main goal of this thesis was to provide recommendations on semen analysis and IUI related laboratory procedures, to optimize diagnosing infertility, counseling of the couples and treatment success. We showed, despite several uncertainties and difficulties, numerous points for improvement. Standardization of used procedures is the major factor of these. At the moment, realizing short-term standardization of semen analysis and IUI treatment is an unrealistic ambition. Providing tools for realizing a step-by-step approach is the best we can do. Also, since the willingness to implement recommendations provided in IUI guidelines is rather low (12,13), it is important to pay attention to selecting adequate implementation strategies. Therefore, the potential clinical areas for clinical effectiveness activities, the likely benefits and costs required to introduce guidelines and the likely benefits and costs as a result of any changes in provider behavior need to be considered (94). Recommendations based on theory-based research should provide citations to original literature related to theory-based research, as well as methodological details regarding the way recommendations have been operationalized and analyzed (95).

Interestingly, a lot is still unknown, even though IUI has been performed for a long time. Technical aspects of semen analysis and IUI with missing evidence or a low level of evidence are not exceptional. Where literature is scarce, findings of each new study can have significant impact on earlier provided recommendations. Preferably, large standardized RCTs should be performed evaluating the impact of IUI-related factors on IUI outcome. Once these new studies will result in updated recommendations, it is important to pay special attention to implementation of this on IUI laboratories. If laboratories are already willing to adapt their laboratory procedures, we would recommend them to sustain an ejaculatory abstinence of up to 3 days (96,97), avoid long time intervals between semen production and processing (98) and use zwitterion-buffered media (99). These methods were found as best practice in both our literature review (chapter 2) and questionnaire study (chapter 3).

Among the semen parameters assessed during semen analysis, most useful improvement could be reached for morphology assessment. As shown, the reliability of the assessment might be improved by using an easier staining method, better and more meaningful agreements in defining normal and abnormal spermatozoa or improving computer assisted semen analysis. For now, it is still useful to assess sperm concentration, morphology and motility during semen analysis and use the results during counseling of the infertile couple. In couples suffering from male factor infertility, IUI should especially be performed in couples categorized as mild to moderate. The value of IUI for couples with unexplained infertility could be subject for further evaluation.

Important requisites to reduce practice variation and variability of semen analysis results are quality control programs and training of the laboratory technicians. It might be useful to take sensemaking into account during development of training programs. Also, the BSAC of ESHRE should be used as guideline for background theory. After initial training, knowledge levels should be maintained, for example by internal and EQC programs, refresher courses or video instructions. It is also important that findings of EQC programs are reported to participants, providing information on performance of the individual semen assessment results over time, as well as compared to other laboratories.

Preferably, laboratory specific programs are developed combining implementation strategies of provided (evidence-based) recommendations, step-by-step realization of standardization, quality control programs (both internal and external) and adequate training of the personnel.

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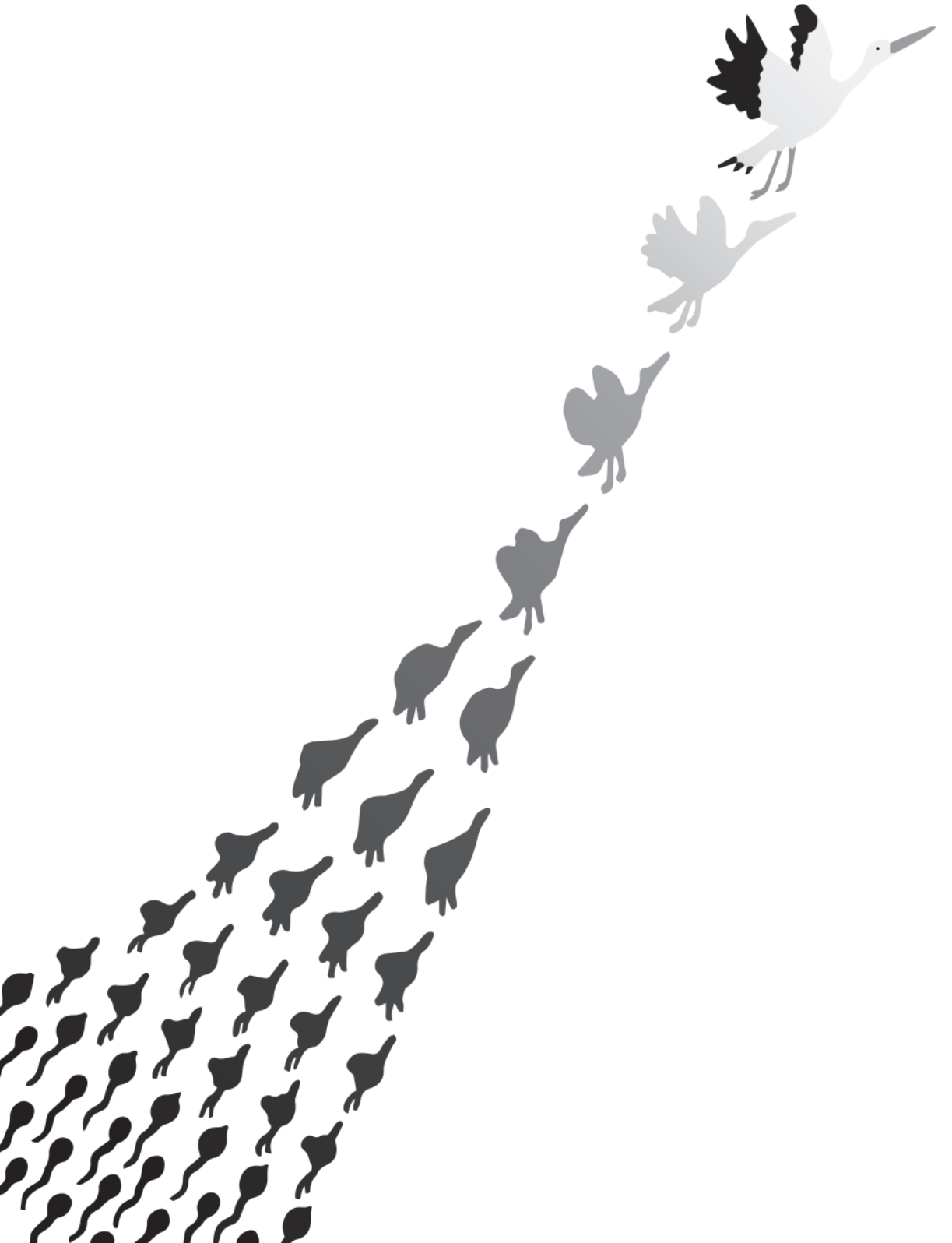


CHAPTER 8

Summary / Samenvatting

Summary

Approximately 15% of the reproductive couples suffer from infertility, of which about 50% is due to male factor infertility. The development of spermatozoa during spermatogenesis and, eventually, the fertilization of an oocyte are complex processes. Several factors can affect these processes, causing defects in the production or transport of spermatozoa. During semen analysis sperm concentration, morphology and motility are assessed, which provides useful information for the evaluation of male fertility. Moreover, this information is relevant for selecting the correct treatment option for couples affected by male factor infertility. Intrauterine insemination (IUI) is one of these treatment options and is recommended as first-line therapy in couples with mild to moderate male factor infertility. Both semen analysis and IUI are performed on a large scale, however, there are also shortcomings related to them.

This thesis describes the studies that we have performed in order to provide tools and recommendations to infertility clinics to improve laboratory aspects of semen analysis and IUI results in couples with male factor infertility.

One of the shortcomings of both semen analysis and IUI is the lack of standardization of used methods. In order to improve standardization, it is important to increase knowledge on the subject and to provide evidence-based recommendations. As a starting point, **Chapter 2** examines the impact of the procedures and equipment used during semen collection, semen processing and insemination on pregnancy rates of IUI. The available literature was reviewed and findings were compared with the laboratory manual of the World Health Organization (WHO). For several procedures, the level of evidence of the provided recommendations was rather low or the recommendations were characterized by insufficient supportive literature. Even though evidence is poor, some recommendations were provided other than the recommendations provided by the WHO. Furthermore, we showed that there are opportunities for future research to provide better evidence-based recommendations.

Where chapter 2 provides an overview of the available literature, we evaluated in **Chapter 3** the laboratory procedures used during IUI in Dutch fertility laboratories and the impact of these procedures on the pregnancy results of IUI. A questionnaire was developed to obtain information on methods used during both semen analysis and IUI and requested ongoing pregnancy results of IUI. About hundred Dutch fertility laboratories were invited to participate, 49 of them completed the questionnaire. It turned out that the lack of standardization reported in previous studies, was also seen in the Netherlands, especially for methods used during semen analysis. Specific laboratory IUI methods were reported to have a significant effect on the probability to become pregnant, in agreement with the

best practice findings reported in chapter 2, i.e. semen collection place, semen preparation technique and method of timing IUI. The discriminatory accuracy of the multivariable model was low (AUC 0.54). Using cycle specific data would be more accurate, however, the findings confirmed again the need of standardization.

Next to standardization of semen analysis and IUI procedures, it is also of importance that correct counselling of infertile couples takes place. The value of sperm parameters in predicting IUI success is in this matter a useful requisite. **Chapter 4** describes the predictive power of sperm morphology and the total progressively motile sperm count (TPMSC) (assessed during fertility workup) and the number of inseminated progressively motile spermatozoa (NIPMS) (assessed at the time of IUI) for the probability of achieving a pregnancy after IUI. A retrospective, observational study including all couples who visited the fertility laboratory of the Radboudumc for a finished IUI episode was conducted. For predicting the pregnancy chance after a first finished IUI episode, a positive relationship was found for ≤4% of morphologically normal spermatozoa (OR 1.39) and NIPMS of 5-10 million (OR 1.73). When other factors were taken into account as well (such as female age, male age, number of cycles in the episode), only sperm morphology and NIPMS remained significant predictors. This model had a discriminatory accuracy of 0.73 and indicates that IUI is especially of relevance in couples with moderate male factor infertility.

Another point of improvement in the accuracy of standard semen analysis results is reducing the high levels of intra-and inter-laboratory variability. Strategies to reduce variability and the lack of standardization are the implementation of external quality control programs (EQC) and the training of technicians performing the standard semen analysis. **Chapter 5** describes the impact of both EQC and training over time on the variability of semen analysis results of Dutch laboratories. All participants of the Dutch EQC program analysed 144 semen samples for sperm concentration and morphology (2001-2018) and 48 samples for motility assessment (2013-2018). A decreasing trend for inter-laboratory coefficients of variation (CVs) was shown for sperm concentration assessment in the total period (24.0-97.5% in 2001-2004 towards 12.7-20.9% in 2015-2018), but such a trend was not found for morphology and motility assessments. Also, we evaluated the short-term effects of training using the results of 19 basic semen analysis courses offered in the period 2008-2018. For all semen parameters, CVs decreased significantly from pre-test to training measurements ($p<0.05$). The CVs of the examination measurements were also significantly lower than pre-test measurements for morphology ($p<0.01$) and motility ($p=0.04$) assessments.

Positive immediate effects of training were not only reported in chapter 5, but also in other studies. The long-term impact of training of technicians on the variability of

semen analysis results, however, was not studied before. **Chapter 6** describes the impact of long-term effects on semen analysis results in the Dutch EQC program, by offering an on-site training based on recommendations provided by the WHO and ESHRE. This on-site training was developed, since Dutch fertility laboratories reported the need for additional semen analysis training opportunities. We compared the results of four pre-training measurements (assessed in 2015) with four post-training measurements (assessed in 2016). There were no statistically significant differences between semen analysis results of the 8 laboratories included in the intervention group compared to the laboratories without training. Also, the performance score assigned by the EQC program was comparable between both groups: 72% (intervention group) vs. 78% (control group) of the maximum achievable score.

In **Chapter 7**, we discuss the main findings of our studies. We showed that the lack of standardization related to semen analysis and the technical phase of IUI is still a major problem. More evidence-based recommendations, together with adequate implementation strategies are needed to improve guideline implementation. Also, the subjectivity of the analysis of semen parameters is a factor of concern. The reliability of the semen parameter assessment should be improved, in order to select the best treatment option for infertile couples. Useful strategies are EQC programs and training of technicians, since they result in reduced variability of semen analysis results. Based on the findings in this thesis, the development of laboratory specific programs is recommended, including implementation strategies, evidence-based recommendations, quality control programs and training of the personnel.

Samenvatting

Ongeveer 15% van de koppels heeft te maken met verminderde vruchtbaarheid, zo'n 50% daarvan wordt veroorzaakt door een mannelijke factor. De ontwikkeling van zaadcellen vindt tijdens spermatogenese plaats en is net als de bevruchting van de eicel een complex proces. Er zijn verscheidene factoren die het kunnen verstoren, waardoor er een defect kan ontstaan bij de productie en/of het transport van de zaadcellen. Tijdens de standaard semenanalyse worden de concentratie, morfologie en motiliteit van de zaadcellen bepaald. Dit levert belangrijke informatie op om de mannelijke vruchtbaarheid te kunnen bepalen. Deze informatie is ook belangrijk bij de keuze van de vruchtbaarheidsbehandeling voor een koppel met vruchtbaarheidsproblemen. Intra-uteriene inseminatie (IUI) is een van die behandelmogelijkheden en wordt aangeraden als eerstelijnstherapie voor koppels met een milde tot matige mannelijke factor. Er zijn verscheidene gebreken bekend van de standaard semenanalyse en IUI, ondanks dat beide op grote schaal worden uitgevoerd.

In dit proefschrift worden de onderzoeken beschreven die we hebben uitgevoerd om handvatten en aanbevelingen aan vruchtbaarheidsklinieken te bieden, om op die manier vanuit laboratorium perspectief de resultaten van semenanalyse en IUI bij koppels met een mannelijke factor te verbeteren.

Het gebrek aan standaardisatie is een groot probleem bij zowel semenanalyse als IUI. Om dit te verbeteren, is het belangrijk dat er voldoende kennis beschikbaar is en dat aanbevelingen gebaseerd zijn op feiten. Daarom werd er in **Hoofdstuk 2** onderzocht wat de invloed is op de zwangerschapskans van IUI van het gebruik van bepaalde procedures en apparatuur gebruikt tijdens de collectie, verwerking en inseminatie van zaad. Daartoe is de beschikbare literatuur beoordeeld en werden de bevindingen naast de laboratorium handleiding van de Wereldgezondheidsorganisatie (WHO) gelegd. Er waren verschillende procedures met een laag level of evidence voor de geleverde aanbevelingen of deze werden amper bevestigd in de gevonden literatuur. Toch gaven we een aantal aanbevelingen die anders waren dan beschreven door de WHO in hun laboratorium handleiding. Ook werden er een aantal punten benoemd die in toekomstig onderzoek bekeken kunnen worden om tot betere aanbevelingen te komen.

In **Hoofdstuk 3** werd bekeken welke laboratorium procedures er gebruikt worden in Nederlandse fertiliteitslaboratoria. Ook onderzochten we wat de invloed van deze factoren was op de zwangerschapskans bij IUI. Er werd een vragenlijst ontwikkeld waarin informatie werd opgevraagd over de methodes die de laboratoria gebruikten bij semenanalyse en IUI. Ongeveer 100 fertiliteitslaboratoria werden uitgenodigd om de vragenlijst in te vullen, 49 daarvan vulden de vragenlijst volledig in. Ook in Nederland was er sprake van een gebrek aan standaardisatie, met name voor

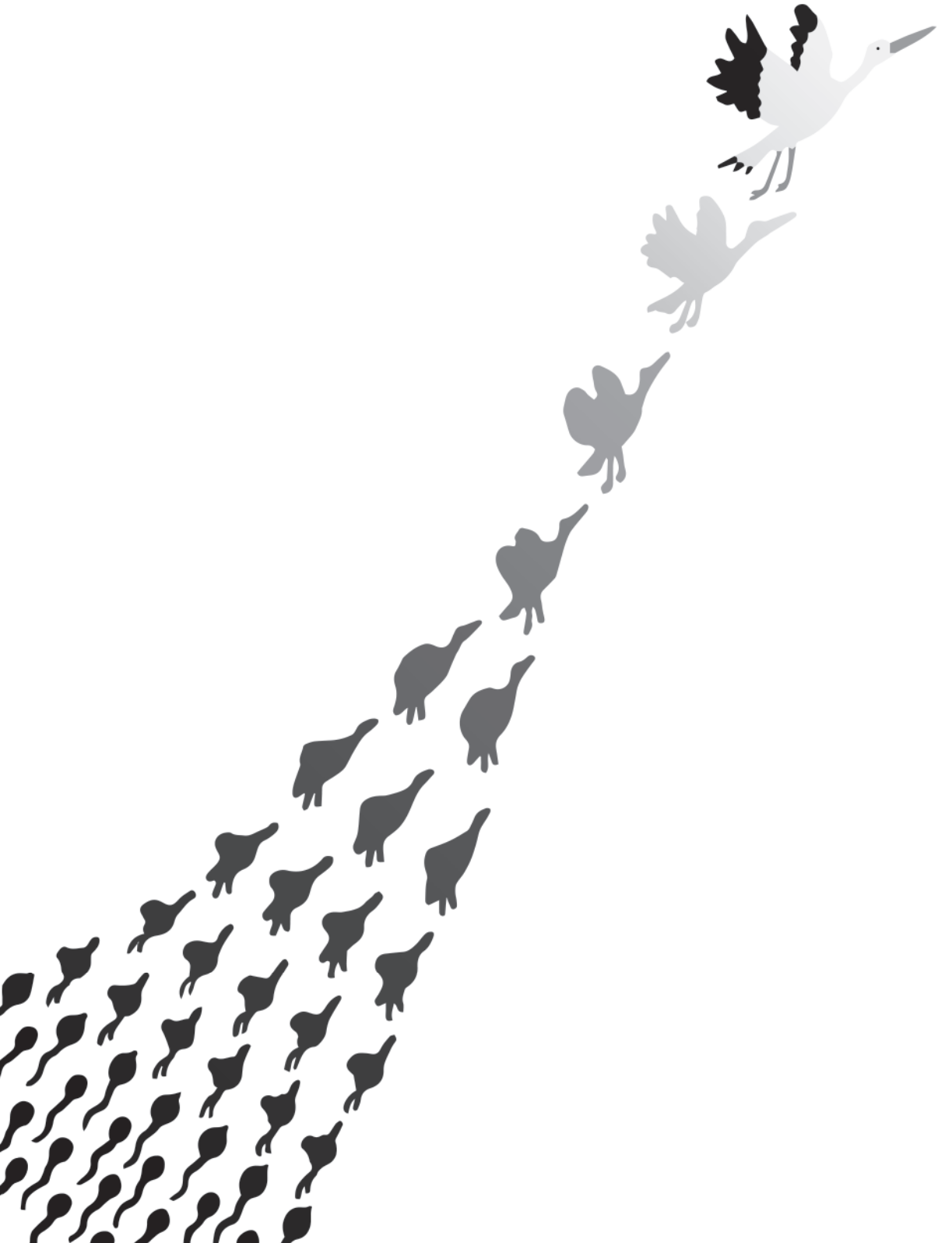
de methodes gebruikt bij semenanalyse. Voor sommige IUI methodes werd een significante invloed op de zwangerschapskans gevonden, overeenkomend met de resultaten gevonden in hoofdstuk 2, namelijk de plaats waar het zaad werd verzameld, de zaad opwerk methode en de methode waarmee IUI getimed wordt. De nauwkeurigheid van het multivariabele model was laag (AUC van 0.54). Wanneer data specifiek per cyclus bekeken zouden worden, zou dit meer accurate resultaten opleveren. Toch werd opnieuw bevestigd dat standaardisatie belangrijk is.

Niet alleen standaardisatie van semenanalyse en IUI is belangrijk, maar ook dat de counseling van koppels met vruchtbaarheidsproblemen juist is. Het is daarom belangrijk dat de zaad parameters die tijdens semenanalyse bepaald worden een goede voorspellende waarde hebben om IUI succes te kunnen bepalen. **Hoofdstuk 4** beschrijft daarom de gevonden voorspellende waarde van de gemeten concentratie, morfologie en motiliteit van het zaad. De morfologie en het totale aantal progressief motiele spermatozoa (TPMSC) werden bepaald tijdens het fertiliteitsonderzoek en het totale aantal geïnsemineerde progressief motiele spermatozoa (NIPMS) werd bepaald op het moment van IUI. In deze retrospectieve studie werden alle afgeronde IUI episodes meegenomen die waren uitgevoerd in het Radboudumc. Een waarde van ≤4% morfologisch normale zaadcellen (OR1.39) en een NIPMS van 5-10 miljoen (OR 1.73) bleken een positieve relatie te hebben met de kans op een zwangerschap na een eerste afgeronde IUI episode. Wanneer andere factoren, zoals de leeftijd van de vrouw en man en het aantal cycli in de episode, ook werden meegenomen, bleven de morfologie en NIPMS significant voorspellende factoren. Dit model had een AUC van 0.73, wat aangeeft dat IUI vooral bruikbaar is voor koppels met een milde tot matige mannelijke factor.

Een ander verbeterpunt voor de standaard semenanalyse procedure is het verminderen van de gerelateerde intra- en inter-laboratorium variabiliteit. Om deze variabiliteit te verminderen en de standaardisatie te verbeteren, kan een extern kwaliteitscontrole (EQC) programma worden ingevoerd of training van het laboratoriumpersoneel plaatsvinden. **Hoofdstuk 5** beschrijft de invloed van een EQC programma en van training op de variabiliteit van semenanalyse resultaten van Nederlandse laboratoria. De deelnemers van het Nederlandse EQC programma beoordeelden van 144 zaadmonsters de concentratie en morfologie (2001-2018) en van 48 zaadmonsters de motiliteit (2013-2018). De inter-laboratorium variatiecoëfficiënt (CV) nam langzaam over de tijd af voor de concentratie bepalingen (van 24.0-97.5% in 2001-2004 naar 12.7-20.9% in 2015-2018). Dit was niet het geval bij de bepalingen van morfologie en motiliteit. Ook werd het korte termijneffect van training geëvalueerd, op basis van 19 semenanalyse trainingen aangeboden in de periode 2008-2018. De CVs namen significant af voor alle parameters tussen pre-test en training bepalingen en voor de morfologie ($p<0.01$) en motiliteit ($p=0.04$) ook tussen de bepalingen van de pre-test en het examen.

De positieve korte termijneffecten van training werden in hoofdstuk 5 en in andere studies beschreven. Het lange termijneffect van training werd echter nog niet eerder beschreven. Daarom wordt in **Hoofdstuk 6** beschreven wat de invloed van een training op locatie op de lange termijn was. Er werd een korte training ontwikkeld op basis van de WHO en ESHRE aanbevelingen en aangeboden aan de deelnemers van het Nederlandse EQC programma. Deze training werd onder andere ontwikkeld naar aanleiding van de vraag van deelnemers voor extra semenanalyse training. De resultaten van vier pre-training metingen in 2015 werden vergeleken met vier post-training metingen in 2016. We vonden geen statistisch significante verschillen tussen de resultaten van de 8 laboratoria met training ten opzichte van de 16 laboratoria in de controlegroep. Ook de score die tijdens het EQC programma aan de deelnemers wordt gerapporteerd was niet verschillend tussen de twee groepen: 72% van de maximaal te behalen score bij de interventie groep versus 78% bij de controlegroep.

In **Hoofdstuk 7** bespreken we de hoofdresultaten van onze studies. We lieten zien dat het gebrek aan standaardisatie bij semenanalyse en IUI nog steeds een groot probleem is. Er zijn meer aanbevelingen op basis van feiten en adequate implementatie strategieën nodig om de implementatie van handleidingen te realiseren. Een anders aandachtspunt is de subjectiviteit van semenanalyse. Het is belangrijk dat de betrouwbaarheid van de semenanalyse resultaten verbetert, zodat de beste behandelmethode voor het koppel met vruchtbaarheidsproblemen geselecteerd kan worden. EQC programma's en training van het laboratoriumpersoneel zijn hiertoe belangrijke strategieën, aangezien ze zorgen voor een afname van de variabiliteit van semenanalyse resultaten. Tot slot valt aan te bevelen om een laboratorium specifiek programma te ontwikkelen, waarbij implementatie strategieën, evidence-based aanbevelingen, kwaliteitscontrole programma's en training van het personeel in acht worden genomen.



CHAPTER 9

Appendix

List of publications

L. Lemmens, S. Kos, C. Beijer, D.D.M. Braat, W.L.D.M. Nelen, A.M.M.Wetzels, for section semen of the Dutch Foundation for Quality Assessment in Medical Laboratories. Technical performance of intrauterine insemination: is it time for a change? Human Reproduction. 2017; 32 (9): 1835–1845

L. Lemmens, S. Kos, C. Beijer, D.D.M. Braat, M.A.Jonker, W.L.D.M. Nelen, A.M.M. Wetzels. Optimization of laboratory procedures for intra uterine insemination: survey of methods in relation to clinical outcome. Andrology. 2018; 6 (5): 707-713

L. Lemmens, S. Kos, C. Beijer, J.W. Brinkman, F.A.L. van der Horst, L. van den Hoven, D.C. Kieslinger, N.J. van Trooyen-van Vrouwerff, A. Wolthuis, J.C.M. Hendriks, A.M.M. Wetzels, for section semen of the Dutch Foundation for Quality Assessment in Medical Laboratories. Predictive value of sperm morphology and progressively motile sperm count for pregnancy outcomes in intrauterine insemination. Fertility and Sterility. 2016; 105 (6): 1462-1468

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Lieve (oud-)Akrissers, al tijdens mijn studententijd werd ik lid van Akris en dat ik nu nog steeds lid ben, geeft wel aan hoe goed ik me op mijn gemak voel bij jullie. Het gaat allang niet meer alleen om tafeltennis, daarmee zou ik de vele avondjes stappen, knotsgekke activiteiten, Akrisweekenden, Corona weerwolven en de fijne samenwerking in alle commissies (en nog veel meer) tekort doen. Het is onmogelijk om iedereen apart bij naam te noemen, zoveel leuke mensen heb ik leren kennen. 'Zuiplui', bedankt dat oma nog steeds overal aan mee mag doen en jullie mij als een echte student laten voelen. Frederick, Maurice, Mianne, Stijn en Thomas, ik had de eer om samen met jullie in het bestuur te zitten. Helaas ligt

het door corona even stil, maar hopelijk kunnen we snel weer onze toffe oud-bestuursuitjes hervatten. Jorien en Mandy, we hebben vaak bij elkaar in het team gezeten, telkens in afwisselende samenstelling. Toch zullen wij altijd, samen met Mariska, team 1 blijven. Laten we dat vooral in stand houden met onze uitjes. Mariska, jij verdient natuurlijk een aparte vermelding. Nadat we ons eerder al bestuursgenoten, teamgenoten en commissiegenoten noemden, mogen we ons nu zeker ook goede vrienden noemen. Of zoals anderen zouden zeggen: Jut en Jul. Bedankt voor alle toffe dingen die we samen doen.

Chris, toen we nog ganggenoten waren, konden we ons soms gedragen als een getrouwd stel. We zien elkaar eigenlijk te weinig sinds jij in Berlijn woont, maar wanneer het wel weer zo ver is, is het meteen als vanouds.

Carola, we hebben elkaar al op verschillende plekken opgezocht: Nijmegen, Steenbergen, Londen, Malmö, Gent en altijd was het reuze gezellig. Bedankt voor al onze leuke belevenissen. Fariza, we leerden elkaar kennen tijdens een snijzaalpracticum, toen bleek dat we dezelfde humor hebben. Dat was voldoende voor een mooie vriendschap, want we slenteren nog steeds samen door Nijmegen, lachen ons rot om elkaars grapjes en kunnen uren achter elkaar spelletjes spelen. Hanneke, het begon allemaal tijdens onze stage, waarbij de onderzoekers van andere kamers aan ons vroegen of het bij ons altijd vrijdagmiddag was. Bedankt voor alle middagen en avonden waarbij we onze gezamenlijke hobby uitvoeren (bordspellen) en we zo hartelijk ontvangen worden door jou, Johannes en de kids. Minet, we hebben helemaal niet zo lang samen met elkaar op de opleiding gezeten, maar het klikte direct. Ook toen jij in Rotterdam en Breda (en ik even in Zweden) woonde, wisten we elkaar te vinden (behalve die keer van het Kronenburgerpark) voor toffe uitstapjes. Extra fijn dat je nu in Nijmegen woont en we elkaar nog vaker kunnen zien.

Ilse, Renske en Anne, na het afgelopen jaar zijn we bijna professionals in online meeten en activiteiten ondernemen. Het wordt tijd dat alles weer mag: samen de 4Daagse feesten onveilig maken, uit eten, oud & nieuw vieren (met champagnepong!) of gewoon weer lekker op stap. Ons weekendje Schiermonnikoog, vlak voordat de maatregelen weer werden verscherpt, vond ik een van de hoogtepunten van 2020. Terecht dus dat we ons hebben voorgenomen er een jaarlijks weekendje weg van te maken.

Lieve Gisella en Janou, dat jullie mijn paranimfen zouden worden, was geen moeilijke keuze. Nadat jullie je taak als mijn getuigen met zoveel verve hebben vervuld, was het tijd voor een nieuwe uitdaging. Wat kan ik me gelukkig prijzen met vriendinnen als jullie. Aan onze vriendschap (en onze belevenissen) zou ik met gemak een apart boek kunnen wijden, daar is hier helaas geen ruimte voor.

Ik zou jullie bovendien tekort doen als ik hier probeerde te omschrijven wat jullie zo bijzonder maakt. Dat we (gekscherend) tot elkaars familie zouden behoren, zegt denk ik wel genoeg. Of dat we later als omaatjes samen in een boerderij in Overasselt willen wonen. Bedankt dat jullie er altijd voor me zijn en voor zoveel gekkigheid en 'lachen, gieren, brullen' zorgen. Wellicht is mijn verdediging een mooi moment om de naam van onze appgroep (de drie wijzen) in de praktijk te laten zien.

Oma, Bert, Wilma, Twan, Myrthe en Maurice, jullie zijn een super schoonfamilie. Bedankt voor de nodige afleiding, steun en warmte. Het is fijn om te weten dat jullie altijd voor mij klaar zullen staan. Funs, Saskia, Dain, Skye en Levi, met jullie valt er altijd wat te beleven. Ik kijk altijd met veel plezier uit naar onze gezamenlijke spelletjesavonden of logeerpartijen: gegarandeerd een dolle pret. Funs, je werkte maanden aan een inscape-kist voor ons, dat laat wel zien hoeveel je voor ons over hebt. Dat is geheel wederzijds. Hanny, René, Lasse, Sverre en Olle, bedankt voor de gezellige drukte. Wat is het fijn om toch wat familie dicht in de buurt te hebben (vooral handig voor onverwachte en ongeplande bezoekjes). Het wordt tijd dat de verjaardagsuitstapjes weer hervat kunnen worden! Lou, dankzij jou voel ik me nog steeds af en toe een student. Onze eeuwige strijd om ergens beter in te zijn dan de ander, zorgt gelukkig nog steeds voor hilarische momenten. Bedankt voor de bijzondere band die we hebben.

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Lieve Luc, wat heb jij dit hele traject vaak vervloekt. Ik durfde soms bijna niet te zeggen tot hoe laat ik de avond ervoor had doorgewerkt. Ik weet dan ook niet wie van ons twee blijer is dat dit bijna is afgerond. Toch was daar die onvoorwaardelijke steun, vooral de laatste maanden. Ik zal weer meer tijd krijgen voor alles wat we zo graag samen doen. Voorlopig geen nachtelijke werksessies meer. Bedankt dat je altijd aan mijn zijde staat. Jij laat me ervaren wat echte liefde is. En wat gelukkig zijn betekent.

Lieve, kleine Alex, je hebt nog geen flauw benul van dit alles. Toch was jij de afgelopen maanden natuurlijk de leukste afleiding. Bedankt dat jij me iedere dag op een lieve lach trakteert. Dat is de allerbeste motivatie, voor alles.

Curriculum vitae

Louise Lemmens werd geboren op 10 september 1990 in Heerlen en groeide op in het Limburgse dorp Schimmert. Ze behaalde in 2008 haar VWO diploma aan het Stella Maris College gelegen in Meerssen. Hierna ging zij biomedische wetenschappen studeren aan de Radboud Universiteit Nijmegen.

Tijdens haar bachelor liep ze stage op de afdeling hematologie in het Radboudumc Nijmegen, waar ze kennis maakte met wetenschappelijk onderzoek. Ze besloot te kiezen voor de masteropleiding Epidemiologie, die ze ook aan de Radboud Universiteit Nijmegen volgde. Haar eerste masterstage volgde ze binnen de voortplantingsgeneeskunde onder leiding van dr. W.L.D.M. Nelen, waar ze onderzoek deed naar het ontwikkelen en valideren van predictie modellen voor de kans op een levend geboren kind na een IVF of ICSI behandeling. Na een buitenlands uitstapje naar Malmö in Zweden voor haar afrondende masterstage, behaalde ze in 2014 haar masterdiploma.

Eind 2014 begon ze als wetenschappelijk onderzoeker bij het Radboudumc. Dit onderzoek groeide uit tot een promotietraject onder leiding van prof. dr. D.D.M. Braat (promotor), dr. W.L.D.M. Nelen (copromotor) en dr. A.M.M. Wetzels (copromotor). Het proefschrift dat nu voor u ligt, is daarvan het resultaat.

Sinds begin 2018 combineerde ze haar onderzoek met andere werkzaamheden. Eerst begon ze als junior project manager voor internationale klinische trombose studies bij Elrohe B.V. in Vierlingsbeek. Sinds april 2019 werkt zij als data-analist ter ondersteuning van (farmaco-) epidemiologisch onderzoek bij het PHARMO Instituut te Utrecht.

Louise woont in Nijmegen, samen met Luc (waarmee ze sinds 2018 getrouwd is) en hun zoontje Alex, die in juni 2020 geboren is.